Pathological actions of *STAT4*, *MTOR* and *STAT3* in human T cell differentiation

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STATEMENT OF DECLARATION

The experimental work presented in this thesis constitutes original work by myself carried out at The Canberra hospital and the John Curtin School of Medical Research under the supervision of Prof Matthew Cook, except where due acknowledgement is made in the acknowledgment section.

This thesis follows the Australian National University guidelines and registrations. This work contained within has not been submitted for the purpose of obtaining any other degree at this or other universities.

Jalila Said AlShekaili

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ABSTRACT

There is an expanding catalogue of immune deficiency diseases with inflammatory and autoimmune manifestations. Understanding the genetic basis of this paradoxical association could enhance our understanding of the pathophysiology of not only rare immune deficiency syndromes, but also more common inflammatory or autoimmune diseases.

We aimed to elucidate mechanism of such diseases using two approaches. First, a discovery project in which we identified a proband who presented with both inflammation and recurrent infections, including bronchiectasis by the age of 4 years, which was associated with hypergammaglobulinaemia. Whole exome sequencing revealed novel mutations in *STAT4* and *MTOR*. Until now, there has been no human genetic analysis supported by functional studies to determine the role of STAT4 or MTOR in Th1 effector differentiation.

The *STAT4* mutation (affecting the DNA binding domain) results in prolonged STAT4 phosphorylation and nuclear retention, thus conferring a gain-of-function phenotype. We show that this promotes excessive Th1 and follicular helper T cell (TFH) formation. The *MTOR* mutation affects the negative regulatory domain, and is therefore also gain-of-function. We found biochemical evidence for cross-regulation between STAT4 and mTOR that explained accentuated TFH and Th1 formation. In this case, enhanced

TFH formation might help explain the aberrant antibody response observed in the proband.

The second approach examined T cell differentiation in patients with known defects in STAT3. Loss-of-function (LoF) mutations in *STAT3* result in autosomal dominant hyper IgE syndrome (ADHIES), and these patients present with unexplained atopic manifestations. Mouse studies have revealed that cytokine signalling via STAT3 promotes IL10 production by Th2 cells that have regulatory role. We found that suboptimal STAT3 signalling in humans also compromises formation of inducible IL10 production by human Th2 cells, which might explain this accentuated atopic phenotype in ADHIES.

More recently, patients with STAT3 gain-of-function (GoF) mutations have been reported in which antibody deficiency occurs concurrently with organ-specific autoimmunity. We observed that suboptimal STAT3 signalling promotes CD4+ T cell exhaustion marked by PD1 and CD57 expression. Since CD57+ PD1^{high} cells constitute a significant subset of human TFH cells, we proceeded to characterize this population in detail. Interestingly, we show that this subset contains CD4+ T cells with cytotoxic gene expression signature and activity. Interestingly, cytotoxicity is attenuated in CD57+ TFH cells when compared with their circulating counterparts. STAT3 LoF results in expansion of circulating CD57+ cells, but does not increase the cytotoxic fraction above the proportion normally observed in tonsil. By contrast, STAT3 GoF enhances the cytotoxic fraction in CD57+ CD4+ T cells in blood, with cytotoxic action against autologous B cells.

Since STAT3 is known to promote CXCR5 expression and abundance of TFH cells, concurrent promotion of cytotoxicity within TFH cells might provide an additional mechanism for defects in antibody production within germinal centres of patients with enhanced STAT3 activity. These findings point to events in germinal centres that can explain the paradoxical association between antibody deficiency and autoimmunity.

Taken together, these approaches provide insight into new mechanisms to explain concurrent immune deficiency and autoimmunity.

PUBLICATIONS ARISING

STAT3 regulates cytotoxicity of human CD57+ CD4+ T cells in blood and lymphoid follicles (Alshekaili et al. 2018).

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ABBREVIATIONS

AD	Autosomal Dominant
AD-HIES	Autosomal dominant (AD) hyper IgE syndrome (HIES)
APC	Antigen presenting cell
BCL-6	B-cell lymphoma 6
CCD	Coiled-coil Domain
cDNA	Complementary DNA
CM	Culture Media
Con	Control
CTLA-4	Cytotoxic T-lymphocyte-associated protein 4
CXCR5	C-X-C chemokine receptor type 5

CYBB	Cytochrome b-245 heavy chain
DBD	DNA-binding Domain
DC	Dendritic cell
DNA	Deoxyribonucleic acid
Eomes	Eomesodermin
FACS	Fluorescence –activated cell sorting
FAT	FRAP, ATM, and TRRAP sharing domain
FCS	Foetal Calf Serum
Foxp3	Fork head Box P3
FRB	FKBP12-rapamycin-binding
GAS	Interferon-gamma activated Sequence
GATA3	Trans-acting T-cell-specific transcription factor
GC	Germinal Centre
GoF	Gain of Function
HEAT	Huntington, elongation factor 3
HEPES	4-2-hydroxyethyl-1piperazineethanesulfonic acid
HC	Healthy Control
HIES	Hyper-IgE-Syndrome
ICOS	Inducible T-cell costimulator
IFN-α	Interferon-Alpha
IFN-γ	Interferon -Gamma
IFGR1	Interferon gamma receptor 1
IFGR2	Interferon gamma receptor 2
Ig	Immunoglobulin
IgG	Immunoglobulin G isotype
IgG1	Immunoglobulin G subclass 1
IgE	Immunoglobulin E isotype
IL-2	Interleukin -2

IL-4	Interleukin -4
IL-10	Interleukin-10
IL-17	Interleukin-17
IL-12	Interleukin-12
IL-12B	Interleukin-12 subunit p40
IL-12RB1	Interleukin 12 Receptor Subunit beta 1
IL-21	Interleukin-21
IL-23	Interleukin-23
ISG15	Interferon-stimulated gene 15
IRF1	Interferon Regulatory Factor-1
JAK	Janus kinase
LAC	Leukocyte activation cocktail
LoF	Loss of function
LPS	lipopolysaccharide
MHC I	Major Histocompatibility Complex I
MHC II	Major Histocompatibility Complex II
mTOR	The mechanistic target of rapamycin
N-domain	*N*-terminus domain
NEMO	NF-kappa-B essential modulator
NRD	Negative regulatory domain
PCR	Polymerase Chain Reaction
PD-1	Program Death-1
PD-L1	Program death-1 ligand
PD-L2	Program death-2 ligand
PIAS	Protein Inhibitors of Activated STAT Proteins
PID	Primary Immunodeficiency Disease
PIKK	Phosphatidylinositol 3-kinase related kinase family
PHA	Phytohemagglutinin

pS6	Phosphopho-S6 ribosomal protein (Ser235/Ser236)
pS6K	Phospho-p70 S6 Kinase (Thr389)
pSTAT	Phosphorylated STAT
PTP-BL	PTP basophil like phosphatase
qRT-PCR	Quantitative Real Time-Polymerase Chain Reaction
RA	Rheumatoid arthritis
Rapa	Rapamycin
RT	Room temperature
SH2	Src Homology 2 Domain
SLE	Systemic lupus erythematosus
STAT1	Signal Transducer and Activator of Transcription-1
STAT3	Signal Transducer and Activator of Transcription-3
STAT4	Signal Transducer and Activator of Transcription-4
STAT5	Signal Transducer and Activator of Transcription-5
STAT6	Signal Transducer and Activator of Transcription-6
T-bet	T-box transcription factor
TBS	Tris-Buffered Saline-Tween
TCR	T cell receptor
TGF-β	Transforming growth factor
T naïve	T naïve cells
Tem	T Effector Memory
Temra	T Effector Memory CD45 RA positive
Tcm	T Central Memory
TFH	T Follicular Helper cells
Th1	T Helper -1
Th2	T Helper- 2
Th17	T Helper- 17
Treg	Regulatory T cells

TSC1	Tuberous Sclerosis Complex 1
TSC2	Tuberous Sclerosis Complex 2
WB	Western Blotting
WT	Wild type

CHAPTER 1 : LITERATURE REVIEW

1. General review

1.1 Differentiation of a naïve T cellS into effector T cellS

1.1.1 General introduction

Most peripheral blood T cells express either CD4+ and CD8+. Both CD4+ and CD8+ T cells integrate many signals in a complex process to give rise to different effector and memory CD4+ and CD8+ T cell subsets. Different CD4+ T cell subsets can be recognized by their surface phenotype or commonly by their signature cytokine for each of these subsets. CD4+ T cell subsets include, T helper 1 [Th1] cells, with interferon –gamma (IFN-γ) as its signature cytokine, T helper 2 [Th2] cells, with interleukin -4 (IL-4) as its signature cytokine, T helper 17 [Th17] cells, with Interleukin-17 (IL-17) as its signature cytokine, T follicular [TFH] cells with interleukin- 21 (IL-21) as its signature cytokine. Each of these subsets appears to be an adaptation for different types of immune responses. Therefore, T cell differentiation toward each of these subsets is crucial for mounting the appropriate immune response towards specific class of pathogen. T cell lymphocytes differentiate down one pathway or another as a result of a complex integration process of three main signals; T cell receptor (TCR), accessory molecules and cytokines that converge on signal transducer and activator of transcription (STAT) mediated signals(Santana and Rosenstein 2003).

1.1.2 TCR signals

Signalling through TCR is crucial for T cell survival. TCR signalling is initiated when the TCR is bound to a peptide on major histocompatibility complex (MHC) on the cell surface of an antigen presenting cell (APC). Therefore, factors affecting the TCR repertoire, strength and duration of binding to a given peptide (affinity) and type of MHC control the outcome of the TCR signalling in dictating the fate of the T cell. T cells that fail to express their TCR die gradually (Labrecque et al. 2001) in a similar

way to those present in environment that lack MHC (Labrecque et al. 2001). T cells engineered to express lower numbers of TCR continue to function, although these cells needed higher antigen dose to activate them (Labrecque et al. 2001) and support proliferation and interleukin -2 (IL-2) production.

1.1.3 Accessory molecules

Accessory molecules include both costimulatory and inhibitory molecules. They are surface receptors that are available at the surface of the T cell, the engagement of which modulates the outcome of the TCR signalling controlling further T cell activation and differentiation (reviewed by Chen, L et al (Chen and Flies 2013)). Co stimulatory molecules include CD28 and inducible T cell co stimulatory molecule (ICOS). CD28 is a well-known co stimulatory molecule expressed on the surface of naïve T cells that supports T cell activation through production of IL-2 and T cell differentiation (reviewed by Sharpe, A.H et al (Sharpe and Freeman 2002)). ICOS shares structural and functional similarities (Hutloff et al. 1999) with CD28 but is only expressed on activated T cells upon activation via TCR and CD28 (Sharpe and Freeman 2002).

Inhibitory molecules control the outcome of the TCR signalling. These include program death-1(PD-1) (Ishida et al. 1992) and cytotoxic T-lymphocyte-associated protein 4 (CTLA-4). PD-1 is up regulated rapidly after T cell activation and on engagement to its ligand, program death-1 ligand 1 and 2) (PD-L1 and PD-L2), it inhibits cell cycle progression, IL-2 production and production of other inflammatory cytokines. CTLA-4 is another negative regulator, which competes with CD28 for costimulatory ligands, and inhibits many CD4+ T cell functions, particularly proliferation (Chambers, Sullivan, and Allison 1997).

1.1.4 Transcription factors

Pathological actions of STAT4, MTOR and STAT3 in human T cell differentiation

The progression from a naïve T cell to an effector or memory cell is a complex process in which an interplay between many external signals are conveyed to the T cell to switch on and switch off transcription regulations and genes expression. Activation of transcription factors is under the control of different cytokines. In general, ligation of cytokine receptors by their cytokines leads to tyrosine phosphorylation of cytokine receptor associated Janus kinases (JAK), which then phosphorylate and activate specific signal transducers and activators of transcription (STAT) molecules that dimerize and polymerize. Nuclear translocation of activated STATs causes up-regulation of specific transcription factors and cellular differentiation (reviewed by Shuai, K et al (Shuai and Liu 2003)).

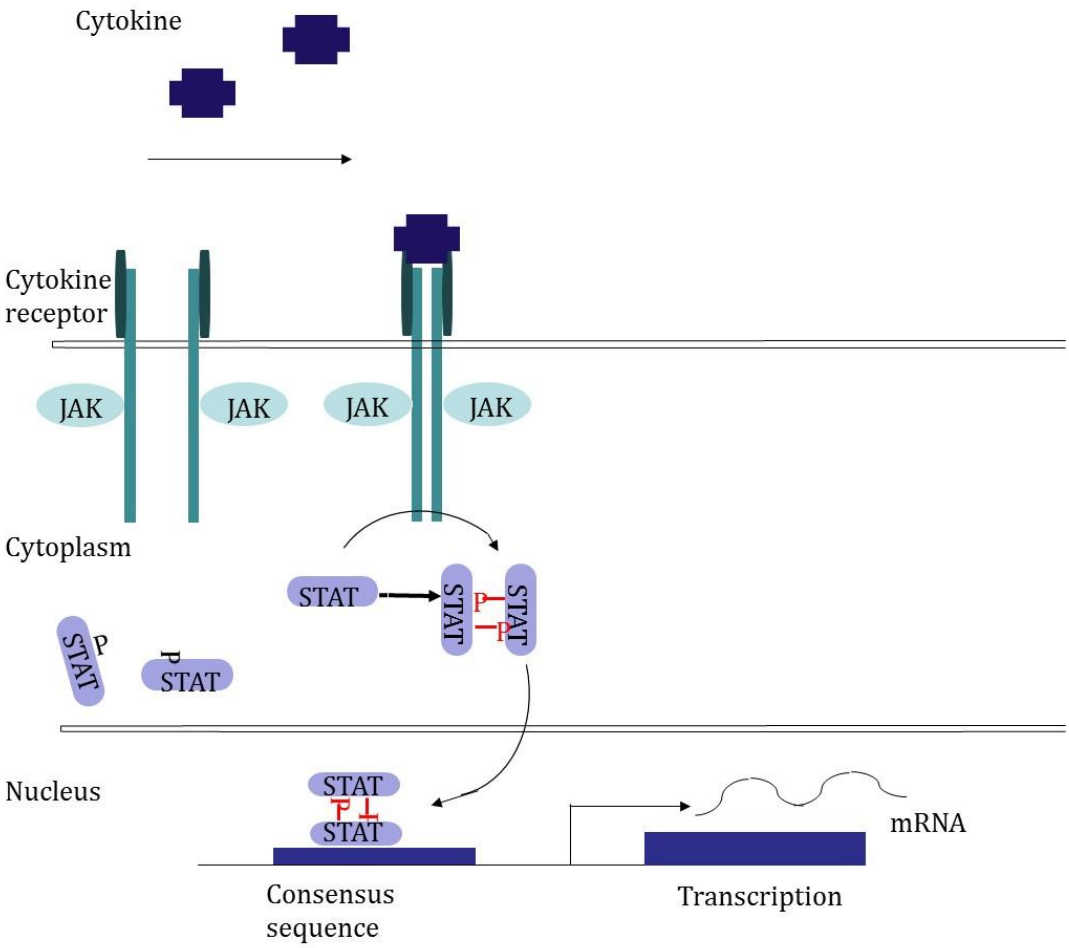

FIGURE 1-1 STRUCTURE AND SIGNALLING OF STAT

Binding of cytokines to their respective cytokine receptors leads to recruitment and activation of JAK molecules. These in turn recruit and activate specific STAT molecules through phosphorylation (P). Activated STAT dimerizes and migrates to the nucleus to bind specific consensus element to activate specific transcription factors.

1.2 Signals needed for differentiation of a naïve T cellS into effector Th1 CELLS

1.2.1 General introduction

T helper-1 (Th1) cells are a distinct effector T cell subset that is characterized by the production of its signature cytokine, interferon- gamma (IFN-γ). The main function of this effector subset is clearance of intracellular organisms through macrophage activation(Hsieh, Macatonia, Tripp, Wolf, Ogarra, et al. 1993). Deficiency of IFN-γ immunity in humans gives rise to a spectrum of diseases characterized by increased susceptibility to mycobacterial diseases (reviewed in (Bustamante et al. 2014)). By contrast, pathological activation of Th1 cells in humans has been iimplicated in organ specific inflammation and autoimmunity (Luckheeram et al. 2012).

The decision of a naïve T cell to undergo Th1 differentiation is under the control of different interrelated signals including TCR, co-stimulation, and cytokines mainly interleukin-12 (IL-12) that activates STAT4 (Tao et al. 1997; Trinchieri 1994; Kaplan, Sun, et al. 1996).

1.2.2 TCR and Th1

Strong T cell signalling favours formation of Th1 measured by IFN-γ secretion (Tao et al. 1997). Moreover, the duration of the TCR activation also influences T cell differentiation in conjunction with strength of TCR signalling (Rogers and Croft 1999). For-example, in short-term stimulation (~4 days), low-level TCR stimulation can support Th1 formation. In contrast, long-term stimulation (~12 days) with strong TCR stimulation was needed to induce formation of IFN-γ producing cells (Rogers and Croft 1999).

1.2.3 Accessory molecules and Th1 cell formation

Co-stimulation is needed to control excessive Th1 deviation and immunopathology associated with Th1 deviation. Deficiency in CD28 (Hunig et al. 2010; Shahinian et al. 1993; Lenschow et al. 1996) or in ICOS (Dong et al. 2001) in animal models was associated with Th1 deviation. However, in rare humans with ICOS deficiency, no specific defect in Th1 formation was observed (Grimbacher et al. 2003a). So far, no human CD28 deficiency has been described.

Inhibitory molecules also regulate Th1 deviation (McAlees et al. 2015). PD-1 deficient mice exhibit increased Th1 cell formation (McAlees et al. 2015), and both auto-inflammatory and autoimmune manifestations (Okazaki and Honjo 2007). So far, no human PD-1 deficiency has been reported, although PD-1 polymorphisms have been reported to segregate with certain autoimmune diseases (ie type I diabetes, and systemic lupus erythematosus) (Nielsen et al. 2003; Prokunina et al. 2002).
CTLA-4 is another inhibitory molecule that is expressed on the surface of activated T cells, and on regulatory T cells (Tregs). CTLA-4 deficient mice exhibit defective regulation of effector T cell proliferation leading to a fatal lymphoproliferative disease associated with increased cytokine release of both Th1 and Th2(Tivol et al. 1995; Chambers, Sullivan, and Allison 1997). Interestingly, there is no haploinsufficiency phenotype in mice. On the other hand, human haploinsufficiency of CTLA-4 results in phenotype that is associated with lymphocyte hyper-proliferation and tissue infiltration (Schubert et al. 2014; Kuehn et al. 2014). This phenotype is similar to that observed in mice with complete CTLA4 deficiency.

1.2.4 Transcription factors and Th1

IL-12 is necessary for Th1 cell formation and cell-mediated immunity. IL-12 receptor ligation activates STAT4 (Hsieh, Macatonia, Tripp, Wolf, O'Garra, et al. 1993). STAT4 activation leads to IFN-γ production which subsequently leads to the up-regulation of the master regulator of Th1 cells, T-box transcription factor (T-bet) and maintenance of the Th1 phenotype (Yang, Ochando, et al. 2007). Furthermore, IFN-γ

activates STAT1 in macrophages and cause further activation and IL-12 production. T-bet up regulation can also be induced via IFN-γ that activates STAT1 in T cells (Yang, Ochando, et al. 2007). Therefore, IL-12(Magram et al. 1996), or STAT4(Kaplan, Sun, et al. 1996)or T-bet (Szabo et al. 2000) deficient mice have deficiency in Th1(IFN-γ production) and have a higher propensity to form Th2 deviated cells (Magram et al. 1996) emphasizing the role of IL-12 STAT4 in initiating and maintaining Th1 differentiation.

In humans, 18 reported inborn errors of Th1 differentiation have been reported so far, summarised in Figure 1-2 (Bustamante et al. 2014), and reviewed by Rosenzweig, S.D. st al(Rosenzweig and Holland 2005)). Defects impeding IFN-γ production include mutations in *IL-12B*, *IL-12RB1*, *ISG15*, *IRF8* and *NEMO* (Bustamante et al. 2014). On the other hand, defects interfering with the function of IFN-γ include mutations in *IFNGR1, IFNGR2, STAT1, IRF8* and *CYBB* (Bustamante et al. 2014). Most of these patients present with increased susceptibility to infections with low-virulence mycobacteria (Rosenzweig and Holland 2005; Doffinger et al. 2002; Bustamante et al. 2014). Interestingly, in contrast to what has been reported in mice (Magram et al. 1996), patients with Th1 defects do not appear to have heightened susceptibility to Th2 associated diseases such as atopy (Wood et al. 2005). Of note, there are few reported mutations in the IL-12/ IFN- γ production pathway in humans similar to the one in mice. However, no *STAT4* or *TBX21* mutations have been reported in humans so far to support or prove their importance for development of Th1 cells in humans.

Pathological actions of STAT4, MTOR and STAT3 in human T cell differentiation

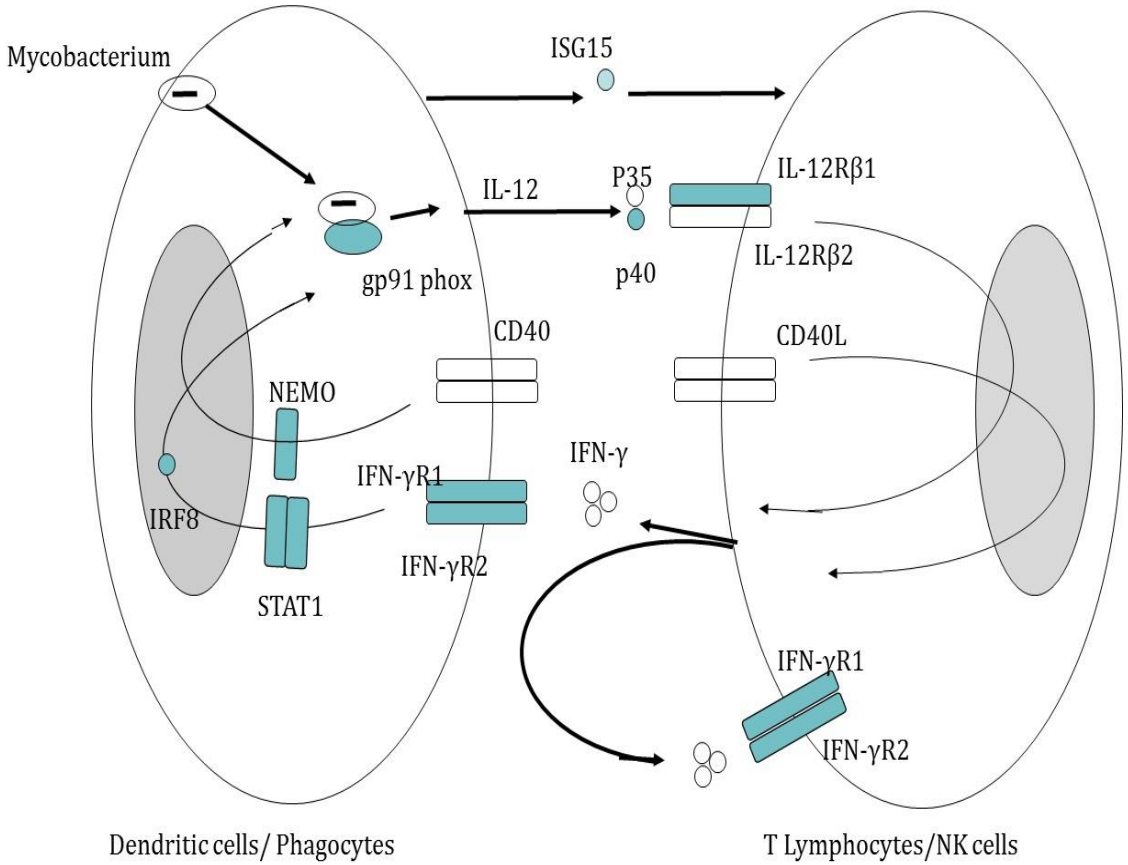

Figure 1-2 Different known mutated proteins causing human inborn error of Th1 differentiation

Outline of the proteins involved in IFN-γ production or action. Mutations in proteins (filled blue colour) have been reported to cause Mendelian susceptibility to mycobacterial disease (MSMD). Mutation of *IL-12B*, *IL-12RB1*, *ISG15*, *IRF18* and *NEMO* interfere with normal IFN-γ production. On the other hand, mutations interfering with action of IFN-γ include *IFN-GR1*, *IFNGR2*, *STAT1*, *IRF8* and *CYBB.* Adapted from (Bustamante et al. 2014).

1.3 Signals needed for differentiation of a naïve T cellS into effector Th2 CELLS

Pathological actions of STAT4, MTOR and STAT3 in human T cell differentiation

1.3.1 General introduction

Th2 responses are important for providing help to B cells for class switching to IgG1 and IgE (Le Gros et al. 1990). Th2 responses are important for immunity against extracellular pathogens.

1.3.2 TCR signalling and Th2 cell formation

The duration and the level of stimulation achieved through TCR activation is also crucial in determining Th2 differentiation (Rogers and Croft 1999). For example, after short-term (~4 days) stimulation of T cells, strong TCR signals gave rise equally to Th1 and Th2 formation (Rogers and Croft 1999). In contrast, after long-term (~12 days) stimulation, a moderate dose of high affinity peptide (strong TCR) is needed to support Th2 formation(Rogers and Croft 1999). In addition, high level expression of major histocompatibility complex II (MHC II) supports Th2 differentiation in mice (Patel et al. 2005).

1.3.3 Accessory molecules and Th2 cell formation

Co-stimulation through CD28 (Hunig et al. 2010; Shahinian et al. 1993; Lenschow et al. 1996) and ICOS (Dong et al. 2001) is needed to support Th2 formation in animal models especially when the TCR signal is weak (Tao et al. 1997; Paul and Zhu 2010) (reviewed by Paul, W.E (Paul and Zhu 2010)). In humans, however, co-stimulation is not mandatory for Th2 formation. Patients with ICOS deficiency have been described and they had normal proportions of Th2 cells compared to normal controls (Grimbacher et al. 2003a). On the other hand, co-inhibitory molecules influence Th2 differentiation in humans. CTLA-4 is crucial in controlling Th2 differentiation in animal models (Oosterwegel et al. 1999). CTLA-4 deficient mice exhibit defective regulation of effector T cell proliferation leading to a fatal lymphoproliferative disease (Chambers, Sullivan, and Allison 1997).

1.3.4 Transcription factors and Th2 cell formation

Cytokines play a major role in shaping Th2 formation as well. Naïve T cell exposure to IL-4 is crucial for inducing Th2 cells (Swain et al. 1990; Seder et al. 1992) through activation of STAT6 (Kotanides and Reich 1996; Schindler et al. 1994) and subsequent up-regulation of Trans-acting T-cell-specific transcription factor, GATA-3 (GATA-3). STAT6 deficient cells failed to undergo Th2 differentiation downstream IL-4 stimulation (Kaplan, Schindler, et al. 1996) and so failed to mediate any of the known IL-4 responses, such as up-regulation of MHC II or IL-4 receptor expression (Kaplan, Schindler, et al. 1996). In contrast, constitutively active STAT6 resulted in induction GATA3 (Kurata et al. 1999) that led to up regulation of c–maf (Ouyang et al. 2000), and Th2 polarization (Kurata et al. 1999; Ouyang et al. 2000). Transgenic STAT6 mice had reduced numbers of CD3+ T cells, which exhibited an activated phenotype and increased propensity to cell death, as well as enhanced Th2 deviation (Bruns, Schindler, and Kaplan 2003; Wurster, Tanaka, and Grusby 2000). Recently, it was also shown that IL-2 acting through STAT-5 has as well a Th2 stimulating effect that is not simply explained by growth induction (Cote-Sierra et al. 2004; Yamane, Zhu, and Paul 2005).

1.4 Signals needed for differentiation of a naïve T cellS into effector Th17 CELLS

1.4.1 General introduction

Th17 cells with their IL-17 as signature cytokine are important for host defences against extracellular organisms, including bacteria and fungi (Annunziato et al. 2007; Harrington, Mangan, and Weaver 2006).

Deficiency of this subset has been described in patients with autosomal dominant hyper IgE syndrome (AD-HIES) (Minegishi et al. 2007; Ma et al. 2008), in which patients are more prone to recurrent bacterial (particularly *S. aureus*) and superficial fungal infections. On the other hand, excessive formation of Th17 cells has been

implicated in many inflammatory conditions. In fact, abnormal expansion of this subset is associated with autoimmunity and lymph proliferation (Milner et al. 2015).

1.4.2 TCR and Th17

High level antigenic stimulation supplemented by low level co-stimulation promotes Th17 differentiation in mice (Bouguermouh et al. 2009). In contrast, human Th17 formation can be promoted in the context of low level of TCR signalling augmented by presence of CD28 (Purvis et al. 2010). Similarly, strong calcium influx compromises the formation of Th17 (Purvis et al. 2010).

1.4.3 Accessory molecules and Th17

Deficiency of ICOS in mouse models did not impede Th17 differentiation from naïve CD4+T cells (Bauquet et al. 2009). However, ICOS is required for the maintenance and expansion of Th17 (Bauquet et al. 2009). In contrast, formation of Th17 cells in humans is severely affected in ICOS deficient patients (Warnatz et al. 2006). In humans, ligation of ICOS leads to induction of Th17 specific transcription factors such as c-MAF, RORC2 and T-bet (Paulos et al. 2010). ICOS is expressed on human naïve CD4+ T cells, but not on mouse naïve CD4+ T cells, which might explain this discrepancy (Paulos et al. 2010).

1.4.4 Transcription factors and Th17

In humans, Th17 formation can be achieved *in vitro* after stimulation of naïve CD4+ T cells in the presence of transforming growth factor beta (TGF-β) and IL-6. This combination activates STAT3 and promotes the up-regulation of transcription factor ROR-gamma (Yang, Panopoulos, et al. 2007) that leads to IL-17 production (Kimura and Kishimoto 2010; Hebel et al. 2011; Qin et al. 2009). IL-23 is then important for the maintenance of IL-17 production in T cells maintained in long term cultures

(Stritesky, Yeh, and Kaplan 2008) (Harrington, Mangan, and Weaver 2006; Langrish et al. 2005).

1.5 Signals needed for differentiation of a naïve T cell into effector TFH

1.5.1 General introduction

T follicular helper cells (TFH) have emerged as a distinct Th subset that resides in the germinal centre (GC) and provides essential help for B cell maturation and differentiation (Kim et al. 2001; Breitfeld et al. 2000; Schaerli et al. 2000). TFH cells are characterized by high level surface expression of CXCR5, ICOS, and PD-1 (Rasheed et al. 2006b) and production of IL-21 that is important for plasma cell formation and immunoglobulin production (Bryant et al. 2007). The formation of TFH is associated with up-regulation of its transcription factor, B-cell lymphoma 6(BCL-6) (Choi et al. 2013).

Cells with a similar but non-identical phenotype are present in the blood, and are sometimes referred to as circulating TFH (cTFH)(Morita et al. 2011). cTFH are memory cells that express intermediate levels of CXCR5 and PD-1 and do not express BCL-6 at levels of GC TFH. Evidence that cTFH are the circulating counterparts of bona fide TFH cells is derived from patients with genetically specified defects in GC formation (Schmitt, Bustamante, and Bourdery 2013; Bossaller et al. 2006). Alteration in cTFH has been shown to be associated with many autoimmune and inflammatory conditions (Choi et al. 2015; Wang et al. 2013).

1.5.2 TCR and TFH

All T cell subsets require TCR signalling for differentiation. The degree or strength of TCR signalling necessary is variable among different T cell subsets. Strong TCR signalling is needed for TFH formation (Tubo and Jenkins 2014; Deenick and Ma 2011)

1.5.3 Accessory molecules and TFH

ICOS is also required for formation of TFH in mice (Bauquet et al. 2009). Similarly in humans, ICOS appears to be important for TFH formation (Grimbacher et al. 2003b) (Warnatz et al. 2006; Bossaller et al. 2006), as patients with ICOS deficiency exhibit deficiency of circulating blood TFH(cTFH) that can be identified by some surface markers such as CD45RO+ CXCR5+. ICOS deficiency also conferred failure of proper GC formation in lymph nodes (Bossaller et al. 2006), further contributing to loss of and defect in TFH formation in these patients.

1.5.4 Transcription factors and TFH

The formation of TFH is associated with up-regulation of its transcription factor, BCL-6 (Choi et al. 2013). However, which signal transducer is involved in this upregulation and therefore formation of TFH subset is still controversial in humans. In mice, IL-21 signalling through STAT3 appears to be crucial for TFH formation (Nurieva et al. 2008). Similarly, in humans, STAT3 appears to be important for cTFH formation (Ma et al. 2012) and therefore possibly TFH formation as well, However, it is interesting to note that in rare IL-21 and IL-21 receptor deficient patients, numbers of cTFH were reported to be normal (Jandl et al. 2017; Stepensky et al. 2015).This might implicated that IL-21 is not crucial for TFH formation. Moreover, *in vitro* studies, suggest that IL-12 is necessary for differentiation of TFH & cTFH from naïve human CD4+ T cells possibly through STAT3 (Ma et al. 2012) (Ma et al. 2009; Schmitt et al. 2009). In addition, patients with IL-12Rβ1 deficiency exhibit abnormal germinal centre formation, although it remains unclear whether TFH function is perturbed (Schmitt, Bustamante, and Bourdery 2013). On the other hand, it has been shown that STAT4 is expressed by human GC TfH cells (Schmitt, Bustamante, and Bourdery 2013), but there is no clear evidence of the role of STAT4 in TFH formation.

2. STAT4

2.1 General introduction

STAT4 protein was discovered based on its homology of the SH2 domain with other STAT SH2 domains (Zhong, Wen, and Darnell 1994; Yamamoto et al. 1994).

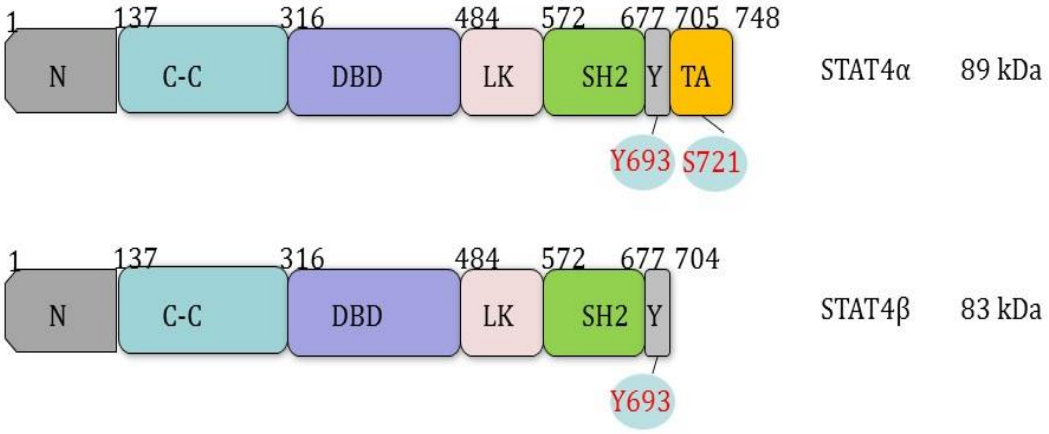

Figure 1-3 Structure of STAT4 and its two isoforms

Different STAT molecules share similar domains; N-domain (N), coiled-coil domain (C-C domain), DNA-binding domain (DNA-BD), linker domain (LK), Src homology 2(SH2) domain and transactivation domain (TA) which contains a critical tyrosine residue (Y) and serine(S) in some STAT molecules. The phosphorylation of the Y as in Y693 in STAT4 is crucial for the STAT4 activation and dimerization through the reciprocal phosphor-Tyr-SH2 domain interaction. There are two isoforms of STAT4; STAT4α and a shorter form, STAT4 β.

2.2 Expression and structure of STAT4

STAT4 expression is restricted to early myeloid precursors (Yamamoto et al. 1994) thymus, testis and spleen (Zhong, Wen, and Darnell 1994). Human T cells express very little STAT4 protein in the resting state. Overall, STAT4 has a similar domain structure to other STAT molecules (reviewed by Levy, D.E. et al (Levy and Darnell 2002)) (Figure 1.3). It is made up of the N-domain which facilitates dimerization of two STAT4 molecules, (Vinkemeier et al. 1998) even when non-phosphorylated (Ota

et al. 2004), and mediates phosphorylation of STAT4 dimers in its anti-parallel conformation (Murphy et al. 2000; Ota et al. 2004). The coiled-coil domain (C-C domain) is necessary for interaction with other proteins. STAT4 forms parallel dimers after phosphorylation, and these bind nuclear DNA regulatory elements via the DNA binding domains (DBD) (Horvath, Wen, and Darnell 1995; Meyer et al. 2003). The SH2 domain is the most structurally conserved domain among STAT proteins. STAT4 docking on cytokine receptors occurs via interaction between the SH2 domain and phosphorylated tyrosine residues on the activated receptor.

2.3 Activation of STAT4

IL-12 is the principal inducer and activator of STAT4. IL-12 receptor is made up of a constitutively expressed IL-12Rβ1 subunit and a IL-12Rβ2 subunit that is upregulated on cell activation. IL-12 itself is the principal cytokine for induction and maintenance of IL-12Rβ2 expression (Wang 2001; Nishikomori et al. 2002; Ramos et al. 2007). As noted earlier (Bacon, Petricoin, et al. 1995), in the resting state, human T cell express very little STAT4 protein. Mitogens such as phytohaemagglutinin (PHA) or T cell activation via TCR and accessory moelcules (e.g. CD2/3/28) up-regulates expression of STAT4 as well as IL-12Rβ2 (Usui et al. 2003; Bacon, Petricoin, et al. 1995; Wang 2001; Nishikomori et al. 2002). STAT4 expression is up regulated 24hrs after stimulation and reaches maximal expression 72 hours after stimulation. T cell activation with IL-12 results in phosphorylation of tyrosine 800 on the IL-12Rβ2, and this forms a docking site for the SH2 domain of STAT4 (Naeger et al. 1999). This interactions leads to STAT4 phosphorylation at tyrosine 693 (Figure 1.1 and Figure 1.3) by JAK2 and Tyk2 (Bacon, Petricoin, et al. 1995; Naeger et al. 1999; Bacon, Mcvicar, et al. 1995). Stimulation with IL-12 phosphorylates STAT4 at tyrosine (693) as early as 5 minutes after stimulation, and this activation can last for more than 30 minutes and perhaps up to 48 hours (Bacon, Petricoin, et al. 1995).

IFN-α can also activate and phosphorylate STAT4 (Cho et al. 1996), although in human T cells, this occurs indirectly though through recruitment of STAT2 (Farrar,

Smith, Murphy, Leung, et al. 2000; Farrar, Smith, Murphy, and Murphy 2000). In contrast, in mice, IFN-α has the ability to activate STAT4 directly (Nguyen et al. 2002). Mutational analysis indicates that the STAT4 N-domain seems to be crucial for its activation by both cytokines (Murphy et al. 2000; Chang et al. 2003). In addition, both IL-12 and IFN-α have the ability to induce IL-12Rβ2 expression and both can phosphorylate STAT4 at both tyrosine 693 and serine 720 (Ramos et al. 2007). It is mainly STAT4 phosphorylated on tyrosine 693 in response to IL-12 or IFN-α that has the ability to bind DNA segments (Cho et al. 1996), including interferon regulatory factor gene (IRF1), which results in IFN-γ production (Yamamoto et al. 1994). Phosphorylated parallel dimers of STAT4 bound to DNA are relatively protected from dephosphorylation (Meyer et al. 2003). This is crucial, since sustained STAT4 activation is necessary for T-box transcription factor (T-bet) up-regulation, which is in turn important for Th1 deviation. T-bet up-regulation appears to be achieved more readily after stimulation with IL-12 than IFN-α (Jacobson et al. 1995; Kaplan, Sun, et al. 1996; White et al. 2001) (Thieu et al. 2008). By contrast, in mice, there is evidence for a vital role for serine phosphorylation for induction of IFN-γ production and Th1 cell differentiation (Morinobu et al. 2002).

2. 4 Isoforms of STAT4

In humans, there are two isoforms of STAT4 protein (Figure 1.3). STAT4β is a protein of 704 amino acids, and lacks the 44 residues at the C terminus of STAT4α (748 amino acids) (Hoey et al. 2003). IL-12 induces tyrosine phosphorylation of both isoforms, which then mediate distinct STAT4 responses. After stimulation with IL-12, STAT4 β maintains its phosphorylation state for longer (>2 hours) and mediates IL-12-dependent cellular proliferation. By contrast, STAT4 α is essential for the formation of Th1 cytokine signature (IFN-γ) in response to IL-12 (Hoey et al. 2003). Over-expression of STAT4β was found to be important in mediating pathological inflammatory diseases (O'Malley et al. 2008; Mo et al. 2008).

2.5 Control of STAT4 activation

Persistent STAT4 activation is important for T-bet up regulation and full Th1 commitment (Thieu et al. 2008). There are many mechanisms that prevent excessive Th1 activation. For example, exposure to IL-12 induces degradation of the total STAT4 protein and phosphoSTAT4 via proteasomal degradation (Wang 2001). The STAT4β isoform is especially prone to proteasomal degradation (Hoey et al. 2003). Activation of proteasomal degradation appears to be specific to the IL-12 system, as IFN-α causes only transient activation of STAT4, and the signal is hardly detectable after 6 hours (Wang 2001). Moreover, after STAT dimers detach from DNA binding sites, the N-domain permits the dimer to assume an antiparallel conformation, which in turn permits dephosphorylation (Mertens et al. 2006) by PTP basophil like (PTP-BL) phosphatase (Nakahira et al. 2007).

2.6 STAT4 and Follicular helper T (TFH) CELLS

TFH cells are distinct CD4+ T cell subset that is terminally differentiated (Rasheed et al. 2006a) and has the ability to support plasma cell formation and antibody production (reviewed by Crotty, S. (Crotty 2011)). Circulating TFH (cTFH) are sometimes used as surrogate markers of TFH in human studies (Gomez-Martin et al. 2011; Ma and Deenick 2014). Recently, it has been shown that in addition to its ability to induce Th1 differentiation through T-bet, STAT4 also also regulates TFH differentiation by inducing expression of both *IL-21* and *BCL-6*. While STAT4 induces Th1 and TFH differentiation states, it appears that the extent of T-bet up-regulation is decisive for CD4+ T cell differentiation, as T-bet act as a repressor for TFH cell differentiation (Nakayamada et al. 2011). Furthermore, it was shown that STAT4 is expressed by GC TFH (Schmitt, Bustamante, and Bourdery 2013) supporting other findings that STAT4 is also important for formation of human TFH (Nakayamada et al. 2011; Schmitt et al. 2009; Wei et al. 2010). Patients with IL-12Rβ1 deficiency have impaired STAT4 signalling. These patients were also found to

have impaired GC TFH formation as well as reduced cTFH and B memory cells (Schmitt, Bustamante, and Bourdery 2013).

2.7 Expression of T-bet

The transcription factor T-bet (encoded by *TBX21*) controls and maintains expression of IFN-γ production, which is a hallmark of Th1 differentiation (Szabo et al. 2000). T-bet is expressed at low levels by naïve T cells, and is up regulated slightly after TCR ligation, (Szabo et al. 2000; Szabo et al. 2002). IL-12, IFN-α and IFN-γ can both up-regulate T- bet expression. Early up-regulation of T-bet is IFN-α and STAT1 dependent during CD4+ T cell activation (Afkarian et al. 2002; Lighvani et al. 2001). The addition of IFN-α, or IL-12 to media containing TCR augments T- bet expression (Ylikoski et al. 2005) through STAT4 activation (Ylikoski et al. 2005; Ramos et al. 2007).

The hallmark of Th1 differentiation is induction of IFN-γ downstream stable T-bet up regulation, which is most efficient in the presence of IL-12 compared to IFN-α or IFN-γ (Ylikoski et al. 2005; Ramos et al. 2007). On the other hand, IFN-α gives rise to transient T-bet up-regulation that reaches its maximum at about 6 hours, and so is insufficient for stable Th1 differentiation in human CD4+ T cells (Thieu et al. 2008; Ramos et al. 2007). Therefore, IL-12/STAT4 act on T-bet to polarize and maintain Th1 differentiation in human CD4+ T cells (Ramos et al. 2007; Usui et al. 2003). In addition, the level of T-bet expression is high in human effector CD4+ T cells compared to the naïve and central memory cell populations(Knox et al. 2014) .

2.8 Diseases associated with abnormal *STAT4*

STAT4 deficiency in mice does not impede T cell development (Hoey et al. 2003) but renders T cells unresponsive to IL-12, which reduces their capacity to up- regulate IRF1, express T-bet, and proliferate (Kaplan, Sun, et al. 1996; Thierfelder et al. 1996; Hoey et al. 2003). As a consequence, STAT4 deficiency in mice results in impaired IFN-γ production and cytotoxicity (Kaplan, Sun, et al. 1996; Thierfelder et al. 1996;

Hoey et al. 2003) in response to IL-12. Moreover, STAT4 deficient CD4+ T cells in mice not only fail to form Th1 cells, but also exhibit a propensity to form Th2 cells (Kaplan, Sun, et al. 1996; Chang et al. 2009). A recent report of DBD variant has been described in association with patients with classic Kaposi sarcoma (Aavikko et al. 2015). The $STAT4^{Thr446Ile}$ variant was associated with lower IFN-γ production, although this difference between mutant and wild type was not statistically significant (Aavikko et al. 2015). There have also been reports of acquired states of human STAT4 deficiency resulting from enhanced degradation of STAT4 protein after chemotherapy(Lupov et al. 2011). In this context, STAT4 deficiency has been reported to reduce Th1 cell formation (Lupov et al. 2011). Finally, a common polymorphism of *STAT4* has been identified to confer increased risk of several autoimmune and inflammatory diseases, including systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA) (Sigurdsson et al. 2008; Zervou et al. 2008; Remmers et al. 2007). This polymorphism was thought to flag an allele with gain-of-function, as it was shown to confer increased sensitivity to IFN-α (Kariuki et al. 2009).

3. Mechanistic target of rapamycin (mTOR)

3.1 General introduction

Rapamycin, named after its discovery in the Easter Island (Rapanui) soil, was first identified as an antifungal that inhibited *Saccharomyces cerevisiae*. It was later found to inhibit cell cycle progression. This led to investigation of the use of the agent as both an antitumor and immunosuppressive drug. The biochemical target is mechanistic target of rapamycin (mTOR) which was initially named the mammalian target of rapamycin (mTOR) (Sehgal, Baker, and Vezina 1975; Vezina, Kudelski, and Sehgal 1975).

3.2 Structure of mTOR

Pathological actions of STAT4, MTOR and STAT3 in human T cell differentiation

The target of rapamycin (TOR) is a conserved ~290 kDa protein that belongs to the phosphatidylinositol 3-kinase related kinase family (PIKK), yet acting as serine/threonine kinase (Brown et al. 1994). mTOR is expressed constitutively in many tissues (Murakami et al. 2004). mTOR has multiple defined protein domains. There are two clusters of huntingtin, elongation factor 3, a subunit of protein phosphatase 2A, and TOR1 (HEAT) repeats within its N-terminal half. Two blocks of HEAT motifs each made up of 40 amino acids, form a pair of antiparallel α helices. The HEAT domains are important in protein-protein interactions, and mediate the nutrient dependent dimerization of the mTOR (Takahara et al. 2006). The HEAT motifs are followed by FRAP, ATM, and TRRAP sharing domain (FAT). The FAT domain is made up of ~500 amino acids and forms a structural scaffold for other proteins (Bosotti, Isacchi, and Sonnhammer 2000). The FAT domain is followed by the FKBP12-rapamycin-binding (FRB). Moreover, the FRB domain is crucial for assembly of the FKBP12-rapamycin complex (Chen et al. 1995). Mutation of serine 2035 to threonine in the FRB domain leads to rapamycin resistance (Dumont et al. 1995). The FRB domain is followed up by serine-threonine protein kinas (kinase) domain which is followed by a negative regulatory domain (NRD). The NRD (residues 2430-2450) is crucial for controlling activity of mTOR. Blockade of this domain by either an inhibitory peptide (Brunn et al. 1997) or deletion of the whole domain (Sekulic et al. 2000b), results in an increased mTOR activity, as assessed by PS6 kinase phosphorylation. At the C-terminal is the FATC domain, which is made up of 31 amino acids and is important for cellular stability and the kinase activity, which appears to be crucial for mTOR activity, as deletion of any of its amino acids compromise the kinase activity of mTOR. FAT and FATC domains interact with each other and this interaction is important for protein kinase activity (Bosotti, Isacchi, and Sonnhammer 2000) (Figure 1.4).

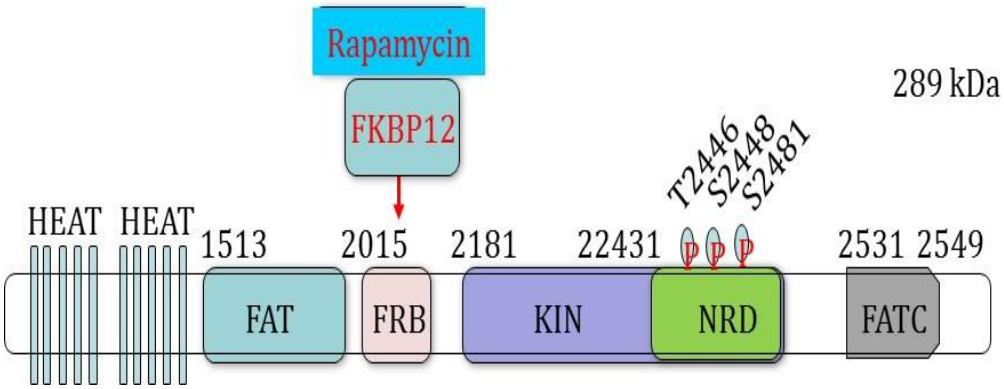

Figure 1-4 Structure of mTOR

The mechanistic target of rapamycin (mTOR) is made up of multiple domains, each serve a different function (detailed in the text). N-terminal domain is made up of huntingtin, elongation factor 3, a subunit of protein phosphatase 2A, and TOR1 (HEAT) repeats. This is followed by FRAP, ATM, and TRRAP sharing domain (FAT). FAT domain is followed by FKBP12-rapamycin-binding (FRB) domain. A serine-threonine protein kinas (KIN) domain is located between FAT domain and a negative regulatory domain (NRD). The NRD (residue 2430-2450) which are followed by FAT domain and FRB domain. At the C terminal there is another FAT domain called FATC domain.

3.3 Function of mTOR

The main function of mTOR is to translate different environmental signals such as availability of nutrients, energy, cytokines, T cell receptor engagement (TCR) and others to control the rate and appropriateness of protein synthesis, cell cycle progression, cell survival and growth. mTOR can associate with other molecules to form two related complexes: mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2), each with different substrates. Most of the known functions of mTOR are due to mTORC1. Extracellular nutrients and insulin stimulate mTORC1 through the phosphoinositide-3-OH kinase PI (3) kinase Akt pathway. Activated Akt phosphorylates the tuberous sclerosis complex (TSC2) on multiple sites, which

removes its inhibitory action on mTOR. Thus, TSC1 and TSC2 act as tumour suppressors. TSC1 and TSC2 are crucial to prevent inappropriate stimulation of mTOR in the face of absent extracellular stimuli, mTOR (Manning et al. 2002; Tee, Anjum, and Blenis 2003; Castro et al. 2003; Garami et al. 2003; Tee et al. 2002; Lee et al. 2007; Qin et al. 2010; Ma et al. 2005). The absence of this normal and basal inhibition of TSC1/TSC2 leads to abnormal growth and division of the cell, as exemplified by defects observed in tuberous sclerosis complex disease, caused by autosomal dominant (AD) mutations in TSC1 or TSC2(Napolioni and Curatolo 2008).

mTORC1 can also be activated via an insulin/PI3K/Akt independent pathway that is mitogen-activated Ras/MEK (MAPK/ERK kinase)/MAPK dependent, causing phosphorylation of the TSC complex to alleviate the inhibitory effect on mTOR. Moreover, mTOR can be activated by TCR ligation, and ligation of the co stimulatory receptor CD28 (Hamilton et al. 2014; Gorentla, Wan, and Zhong 2011; Colombetti et al. 2006). Importantly, mTOR activation is maintained by other signals, including IL-12/STAT4 (Rao et al. 2010). Activation of mTOR leads to phosphorylation and activation of its substrate, S6 kinase (S6K), and phosphorylation and inactivation of the repressor of mRNA translation factor (4E-BP1). Both substrates are sensitive to rapamycin, and both are important for initiating the translational machinery leading up to protein synthesis and cell growth (Laplante and Sabatini 2009; Hay and Sonenberg 2004). Overall, mTOR is activated by the presence of extracellular stimuli through PI3K/ Akt dependent and independent pathways and translates these environmental cues into protein synthesis and cell growth (Figure 1-5).

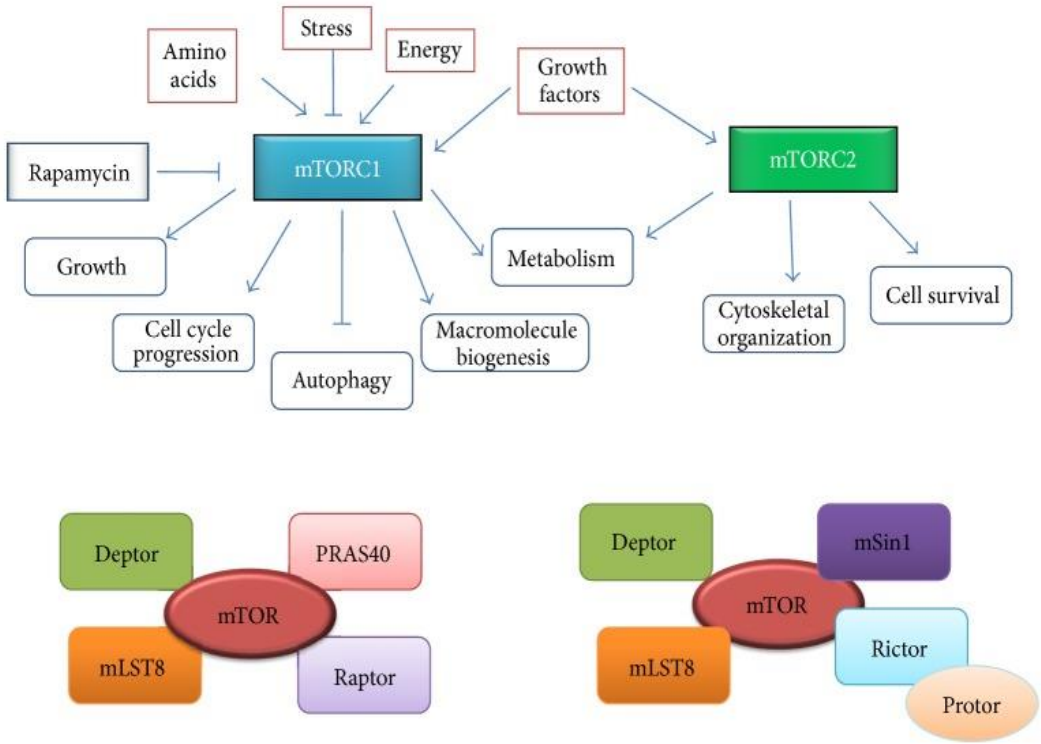

Figure 1-5 Signaling of mTOR

Schematic representation of different stimulants and inhibitors of the mTOR complexes and the downstream effect of mTOR activation into different cellular functions. (Showkat, Beigh, and Andrabi 2014)

3.4 CD8+ T cell subsets

CD45, also known as common leukocyte antigen, is expressed on all human T cells after thymic development (Serra et al. 1988). Two common CD45 isoforms are expressed on T cells. CD45RA is usually expressed on naïve T cells (Prince, York, and Jensen 1992), whereas the CD45RO isoform marks memory (antigen-experienced) T cells (Prince, York, and Jensen 1992). However, there is also a T cell subset that is CD45RA+ despite being antigen experienced that is present among human CD4+ and CD8+ T cells and produces IFN-γ and expresses high levels of perforin (Hoflich et al. 1998). This subset has received the descriptive designation of T-effector memory, CD45RA positive (Temra), and expresses lymph node homing molecules such as CCR7 (CD45RA+CCR-) and CD62L (Sallusto et al. 1999). In

contrast, absence of both CD45RA and CCR7 (CD45RA-CCR7-) defines effector memory T cells (Tem). In summary, human CD8+ T cells can be divided into four subsets using a combination of these surface markers including CD45RA or CD45RO, CD27, CD62L or CCR7 (Hamann et al. 1997; Sallusto et al. 1999; Tomiyama et al. 2004). The relative abundance of each subset in the blood is normally naïve (CD45RA+CCR7+) > Temra(CD45RA+CCR7-) > Tem (CD45RA-CCR7-) > Tcm (CD45RA-CCR7+) (Sathaliyawala et al. 2013). However, human aging is associated with accumulation of the CD8+ T cell Temra subset (Koch et al. 2008). Similarly, chronic viral antigenic stimulation causes accumulation of this subset in humans. Interestingly, mice have three subsets only and lack the Temra subset.

CD8+ T cell subsets defined by surface phenotype are also functionally different. Naïve CD8+ T cells are CD45RA+ and co-express CD27, CD62L and CCR7. They produce mainly IL-2 and lack any cytotoxic activity (Hamann et al. 1997; Sallusto et al. 1999; Romero et al. 2007). They express IL-7 receptor (CD127) which is important for their survival (Schluns et al. 2000; Tuma and Pamer 2002). Expression of (CD127) by naive CD8+ T cells is thought to be important for the expansion phase of the immune response, especially in the context of lymphopenia (Schluns et al. 2000; Goldrath et al. 2002).

CD45RA- CCR7+ CD8+ T cells are defined as central memory cells by virtue of their tendency to home to lymph nodes. They are not cytotoxic *ex vivo* but exhibit recall responses after antigen re-exposure (Hamann et al. 1997; Sallusto et al. 1999). Similar to naïve CD8+ T cells, CD8+ Tcm cells express either IL-7 or IL-15 to maintain their homeostatic proliferation (Schluns et al. 2000; Tuma and Pamer 2002).

Both CCR7 positive CD8+T cells (naïve and central memory) have the capacity to proliferate and divide (Champagne et al. 2001). Moreover, both exhibit high expression level of the pro-apoptotic Bcl-2 molecule (Geginat, Lanzavecchia, and Sallusto 2003) and have also been noted to possess longer telomeres (Sallusto et al. 1999).

Tem and Temra cells are effector memory T cells that have ability to mount effector and cytotoxic responses in the periphery. They produce large amounts of IFN-γ and express perforin (Hamann et al. 1997; Sallusto et al. 1999). Effector CD8+ T cells lack the node homing receptors (CCR7 and CD62L)(Hamann et al. 1997; Sallusto et al. 1999), have shorter telomeres than their naïve counterparts (Sallusto et al. 1999), express only low levels of BCL-2, and exhibit lower proliferative ability and turnover than their memory counterparts (Geginat, Lanzavecchia, and Sallusto 2003). Both Tem and Temra CD8+ T cells express low levels of IL-7 receptor (CD127) (Tuma and Pamer 2002; Geginat, Lanzavecchia, and Sallusto 2003).

The ontogeny of different CD8+ T cell subsets remains contentious, especially in humans. One model describes a linear progression from naïve to Tcm to Tem. Short term TCR stimulation of human naive CD8+ T cells lead them to acquire Tcm phenotype (CD45RA- CCR7+) with little IFN-γ production (Sallusto et al. 1999). Furthermore, short term TCR stimulation of Tcm results in loss of CCR7 and acquisition of inflammatory cytotoxic effector phenotype Tem (Sallusto et al. 1999; Geginat, Lanzavecchia, and Sallusto 2003). Both of these observations support a linear model of naïve to Tcm to Tem in response to short term TCR stimulation. Much less is known about formation of Temra, since mice lack an equivalent subset. Some evidence suggests that Temra can be generated *in vitro* from Tcm by activation in the context of IL-15, while the presence of TCR stimulation antagonises the formation of Temra (Geginat, Lanzavecchia, and Sallusto 2003).

3.5 mTOR and peripheral CD8+ T cell subset differentiation

Recent evidences indicate that mTOR not only inhibits immune system activation, but also also has a somewhat paradoxical action that fine tunes and enhances CD8 immunity. mTOR signalling through the mTORC1 complex influences memory versus effector differentiation of naïve CD8+ T cells (Araki et al. 2009). Blocking mTOR with rapamycin early in the course of viral infection (proliferation phase), or in

the T cell contraction phase, promotes formation of memory CD8+ T cells (Araki et al. 2009), that express high levels of CD127, CD62L and Bcl2 (Tcm, phenotype). These effects appear to be CD8+ T cell-intrinsic, since RNA interference of mTOR solely in CD8+ T cells replicates the effect of incubating PBMCs with rapamycin (Araki et al. 2009). Furthermore, the use of mutant CD8+ T cells, rendered specifically insensitive to rapamycin failed to replicate the phenotype of expanded memory CD8+ T cells when cells were treated with rapamycin (Araki et al. 2009).

3.6 Effect of IL-12- STAT4 and mTOR on CD8+ T cell subset differentiation

IL-12 is important for the differentiation of naïve CD8+T cells into effector and subsequent memory CD8+ T cells formation (Chang et al. 2004). In the presence of IL-12 and antigenic stimulation, naïve CD8+ T cells differentiate into effector cells, producing high amounts of interferon-γ, granzyme-B and acquire more potent cytotoxic activity (Chang et al. 2004; Rao et al. 2010; Chowdhury et al. 2011). Induction of T-bet is crucial in deciding the effector (Knox et al. 2014) over memory(Takemoto et al. 2006) fate of CD8+ T cell subsets after stimulation with IL-12 (Li et al. 2006; Yang, Ochando, et al. 2007). In the absence of IL-12, developing CD8+ T cells exhibit lower expression of T-bet and acquire a memory phenotype (CD127+) (Takemoto et al. 2006).

T-bet up-regulation by IL-12 appears to be STAT4 and mTOR dependent (Rao et al. 2010). Furthermore, mTOR activation (as judged by S6K phosphorylation) was activated for longer in the presence of IL-12, suggesting that these signals are crucial for effector CD8+ T cell differentiation (Rao et al. 2010). Conversely, activation of STAT4 was found to be mTOR-dependent, as the presence of rapamycin reduced STAT4 phosphorylation (Rao et al. 2010) and the absence of active mTOR diminished the ability of IL-12 to phosphorylate STAT4 (Delgoffe et al. 2009). Active IL-12-STAT4 and mTOR were both needed to up regulate T-bet (Li et al. 2006; Takemoto et

al. 2006; Rao et al. 2010) that favour effector fate of CD8+ cells. On the other hand, enhanced quality and increased quantity of Tcm CD8+ T cells was achieved by blocking mTOR leading to down-regulation of T-bet and up-regulation of eomesodermin (Eomes) (Rao et al. 2010). The resulting Tcm were competent to proliferate when restimulated with TCR, and adopted Tem phenotype, expressing high levels of perforin and granzyme-B (Ramos et al. 2009).

3.7 Effect of mTOR on tissue resident CD8+ T cell differentiation

CD8+ T cells are outnumbered by CD4+ T cells in all body compartments including blood and lymph nodes. The exception appears to be in the intestine, where there is an equal distribution of CD4+ and CD8+ T cells (Sathaliyawala et al. 2013). CD8+ T cells at the intestinal mucosa are predominantly tissue resident effector memory cells (Trm) that are recruited to the intestinal mucosa through up regulation of adhesion molecules. These adhesion molecules include CCR9 and α4β7 (Agace 2010), and the level of CD103 expression (Sowell et al. 2014). Temra subsets are not normally present in the intestinal mucosa (Sathaliyawala et al. 2013).

Trm cells have a distinct phenotype that distinguishes them from classical Tem and Tcm. Trm cells were found to be more like effector cells in some aspects; they are $CD62L^{low}$, granzyme-B ^{high}, and $CD69^{high}$, and mount vigorous IFN-γ responses (Sathaliyawala et al. 2013). On the other hand, Trm resemble central memory characteristics in that they have high replicative capacity and high BCL-2 expression (Masopust et al. 2006), which is different from classical effector Tem and Temra population. This phenotype of intestinal tissue resident memory CD8+ T cells is different from those resident in other tissues (Masopust et al. 2006). Once located in the intestine, Trm form long term memory cells that do not recirculate (Masopust et al. 2010).

Up-regulation of tissue homing markers such as CCR9 and α4β7 (Agace 2010), and the level of CD103 expression on Trm CD8+ T cells (Sowell et al. 2014) is under the

control of mTOR (Sowell et al. 2014). While rapamycin enhances the quality and the quantity of CD8+ T central memory, especially at their reservoir in secondary lymphoid organs (SLO), rapamycin impairs the formation of Trm at mucosal sites (Sowell et al. 2014). While there is relatively little data available on the normal distribution of CD8+ T cell subsets in the oesophagus, biopsies from patients with erosive gastritis revealed that, CD8+ T cells as the dominant cell type (De Jonge et al. 2008; Tantibhaedhyangkul et al. 2009). There are very few Temra in the intestine compared to the lung (Sathaliyawala et al. 2013) and the main CD8+ T subset in any tissue including intestine is Trm. Excessive Trm are thought to contribute to the pathogenesis of various inflammatory diseases, including psoriasis (Suarez-Farinas et al. 2011) , allergic contact dermatitis (Gaide et al. 2015) and Crohn's disease (Raine et al. 2015; Kleinschek et al. 2009).

4. Interleukin- IL-10 (IL-10)

4.1 Cellular sources, stimuli and signals required for IL-10 production

IL-10 is produced by many hematopoietic cells including, monocytes, B cells and T cells (Yssel et al. 1992; Xu et al. 2009; Delprete et al. 1993). The importance of haemopoietin sources of IL-10 was demonstrated by the good clinical response to bone marrow transplantation in patients with IL-10 deficiency (Engelhardt et al. 2013; Engelhardt and Grimbacher 2014).

4.1.1 Antigen presenting cells

In mice, IL-10 is produced by monocytes and myeloid dendritic cells in response to different microbial products (Boonstra et al. 2006; Saraiva and O'Garra 2010). Stimulation of pattern recognition receptors such as TLR2 and TLR4 signal in macrophages and myeloid dendritic cells (DC) induces production of IL-10, mainly through MYD88 (Boonstra et al. 2006; Saraiva and O'Garra 2010) (reviewed by Saraiva, M.(Saraiva and O'Garra 2010)). Myeloid DC produce IL-10 in response to

Dectin 1 and TLR2 stimulation through Syk and NF-κB dependent pathways, respectively. On the other hand, plasmacytoid dendritic cells produce little IL-10.

In humans, activation of STAT3 by lipopolysaccharide (LPS) stimulation , is an important but not the exclusive regulator of IL-10 production by human monocytes and human B cells (Staples et al. 2007; Benkhart et al. 2000; Ziegler-Heitbrock et al. 2003). Thus, *STAT3* mutated on tyrosine or lacking DNA binding activity blocks the ability of STAT3 to induce IL-10 production (Benkhart et al. 2000). In addition, IL-10 has the ability to induce its own production by human monocytes (Staples et al. 2007), and this autocrine pathway is entirely STAT3-dependent (Staples et al. 2007).

4.1.2 IL-10 in T cells

In humans, IL-10 can be detected *in vivo* mainly among effector memory (CD45RA-CD4+) T cells after stimulation with TCR co-stimulation with T cell activation and expansion beads (CD2/CD3/CD28)(Cohen et al. 1997; Yssel et al. 1992). Stimulation via common gamma chain cytokines (IL-2, IL-7, IL-4, IL-15, and IL-21) augments IL-10 production by mitogen activated T cells (Cohen et al. 1997). Investigation of rare cases of human deficiency of IL-2Rα (CD25) revealed failure to induce IL-10 by CD4+ T cells after stimulation with mitogen and IL-2 (Caudy et al. 2007). Similarly, IL-10 production by mitogen activated cells is inhibited by rapamycin through inhibition of IL-2 production (Cohen et al. 1997). Interestingly, IL-12 also induces IL-10 from mitogen activated (CD2) human PBMCs and T cells (Meyaard et al. 1996).

4.1.2.1 Th1

In humans, IL-10 can be detected in Th1 clones (Yssel et al. 1992). In mice, memory Th1 cells require continuous exposure to IL-12 to induce IL-10 production and once IL-12 is eliminated from the system, there is a decline in the production of IL-10 (Chang et al. 2007). IL-10 production by murine Th1 requires high concentrations of IL-12 and strong TCR stimulation, and is dependent on active STAT4 and ERK signalling (Saraiva et al. 2009). This appears to provide a regulatory feedback

mechanism that controls Th1 responses while not interfering with proper secondary immune responses. In addition, IL-21 has the ability to augment IL-10 production from murine Th1 cells through STAT3 dependent pathway (Spolski et al. 2009).

IL-12 has also been shown to induce IL-10 from human Th1 clones (Meyaard et al. 1996). Prolonged exposure to IL-12 and concurrent increased IL-10 production is associated with suppression of Th2 effector cytokines in human T cells (Meyaard et al. 1996). IL-10 is not considered to be a memory Th1 cytokine, because in committed Th1 cells, the *IL10* gene is silenced (Kang and Im 2005). However, repeated allergen exposure in human has been associated with the *in vivo* switch of allergen Th1 from IFN-γ producing- to IL-10-producing CD4+ T cells (Meiler et al. 2008). More recently, it was shown that special rare *in vivo* human Th1 subset produces IL-10 (Haringer et al. 2009). IL-10 expression was only observed after continuous and strong stimulation in the presence of IL-12 to maintain its expression.

4.1.2.2 Th2 cells

Human Th2 clones produce more IL-10 than Th1 clones (Delprete et al. 1993). IL-12 has also the ability to induce IL-10 from human mitogen activated (CD2) Th2 clones (Meyaard et al. 1996). IL-4 is known to induce the signature transcription factor GATA3 in Th2 cells (Chang et al. 2007). Repeated exposure of Th2 clones to IL-4 leads to IL-10 production as a stable phenotype of Th2 cells (Chang et al. 2007). In fact, chronic Th2 inflammation in mice results in skewing of Th2 differentiation to cells that make both IL-4 and IL-10. Active STAT3 signalling has a positive effect in inducing more IL-10 production in murine Th2 cells (Altin, Goodnow, and Cook 2012). In mice, this regulatory subset has a gene expression profile that resembles that of regulatory T cells (Tregs), except that they are Foxp3-negative (Altin, Goodnow, and Cook 2012). The subset is characterised by production of both IL 10 and IL-4, and is dependent on STAT6 and GATA3.

4.1.2.3 Th17 cells

Most of the available literatures on IL-10 in Th17 comes from animal models. In the murine system, polarisation to Th17 differentiation is supported by the use of IL-6 and TGF-β, both of which induce STAT3-dependent up-regulation of c-Maf, which in turn induces production of IL-10 (Xu et al. 2009). IL-10 is crucial in controlling Th17-mediated inflammation (Xu et al. 2009). Moreover, IL-21 signalling through STAT3 also augments production of IL-10 in committed Th17 cells in mice (Spolski et al. 2009). The production of IL-10 by Th1, Th2 and Th17 subsets relies on the ERK1/2 and c-maf expression (Saraiva et al. 2009).

4.2 Action of IL-10 and molecules needed to exert its function.

IL-10 is well known as a cytokine synthesis inhibitory factor (CSIF) (Vieira et al. 1991). IL-10 binds to its dimeric receptor which is made up of IL10R1 and IL10R2. IL10R2 is ubiquitously expressed, while IL-10R1 is an inducible subunit. IL-10 binds primarily to IL-10R1 compared to IL-10R2 (von Lanzenauer et al. 2015; Liu et al. 1994), (reviewed by Moore, K.W (Moore et al. 2001)). Differential expression of these two receptors, particularly IL-10R1, determines the extent of responsiveness to IL-10 (von Lanzenauer et al. 2015; Moore et al. 2001). In humans, IL-10R1 is mainly expressed on hematopoietic cells including monocytes, B and T cells (Liu et al. 1994).

IL-10 has a direct suppressive effect on proliferation of naïve and memory T cells (Haringer et al. 2009) as well as directly inhibiting cytokine productions by Th1 cells (Delprete et al. 1993). IL-10 has also the same inhibitory effect on human Th2 clones (Delprete et al. 1993; Altin, Goodnow, and Cook 2012). Recently, it was shown that IL-10 has the ability to inhibit Th2 proliferation directly in a Foxp3 independent pathway (Altin, Goodnow, and Cook 2012) (reviewed by Hawrylowicz, C.M. (Hawrylowicz and O'Garra 2005)). Therefore, IL-10 is crucial in controlling ongoing Th1 immune response and excessive Th2 inflammatory response to prevent

immunopathology associated with exaggerated Th1 or Th2 immune response (Hawrylowicz and O'Garra 2005; Akdis et al. 1998; Altin, Goodnow, and Cook 2012).

4.2.1 Monocytes and IL-10

Much of the available information on effects of IL-10 is derived from studies on antigen presenting cells such as monocytes, macrophage and dendritic cells. Most of the known effects on IL-10 on monocytes are inhibitory. Macrophages activated in the presence of IFN-γ or LPS express high levels of activation markers such as MHC class I and II, as well as B7 co stimulatory molecules (Ding et al. 1993). One of the main actions of IL-10 on monocytes is down-regulation of antigen presentation by macrophages (Vieira et al. 1991) and dendritic cells. This is achieved through down-regulation of MHC class II (de Waal Malefyt et al. 1991; Ding et al. 1993; Buelens et al. 1995), CD80 (Ding et al. 1993), and CD86 (Buelens et al. 1995)expression. IL-10 also inhibits endogenous monocyte inflammatory cytokine production, including IL-1β, IL-6 and TNF-α, which are usually produced early (4-8 hrs) after activation of monocytes (de Waal Malefyt et al. 1991; Fiorentino, Zlotnik, Mosmann, et al. 1991).

IL-10 down regulation of activated antigen presenting cells(Delprete et al. 1993) causes eventually inhibition of T cell proliferation (Fiorentino, Zlotnik, Mosmann, et al. 1991; Fiorentino, Zlotnik, Vieira, et al. 1991; Meyaard et al. 1996) and cytokine production of Th1. In fact, treatment of dendritic cells with IL-10 also reduces their capacity to induce competent T cell responses. Therefore, one consequence of IL-10 treated dendritic cells is induction of Foxp3 positive regulatory cells (induced Tregs) (Steinbrink et al. 2002).

In macrophage and dendritic cells, IL-10 signals mainly through STAT3 (Finbloom and Winestock 1995). Thus, mice engineered to carry targeted STAT3 deficiency in their gastrointestinal macrophages and polymorph nuclear cells (PMN) develop early-onset and severe colitis, a similar phenotype to those mice with IL-10 deficiency (Takeda et al. 1999). Disruption of *STAT3* results in the generation of inflammatory

antigen presenting cells (Cheng et al. 2003; Giacomelli et al. 2011). This explain why monocytes obtained from patients with autosomal dominant hyper IgE syndrome (ADHIES), in which STAT3 signalling is impaired, generate enhanced inflammatory cytokine responses after exposure to LPS (Giacomelli et al. 2011). The main anti-inflammatory action of IL-10 on monocytes and polymorphonuclear leukocytes (PMN) in the gastrointestinal tract is mediated by STAT3 (Takeda et al. 1999), and this appears to be crucial for protection against inflamed bowel. This IL-10 anti-inflammatory response is inhibited by pro-inflammatory cytokines such as IL-1 and TNF-α (Ahmed and Ivashkiv 2000). Inhibition is at the level of STAT3 phosphorylation and subsequent DNA binding (Ahmed and Ivashkiv 2000).

4.2.2 B cells and IL-10

In contrast to the general inhibitory direct and indirect effect of IL-10 on monocytes and T cells, IL-10 signalling through STAT3 has stimulatory effect on B cell, and augments formation of plasma cells (Choe and Choi 1998; Yoon et al. 2009). IL-4, has proliferative effect on B cells but, do not support formation of plasma cells (Choe and Choi 1998). IL-4 supports class switching to IgE production (Punnonen et al. 1993). On the other hand, IL-10 stimulates B cells toward formation of plasma cells in the germinal centres (Yoon et al. 2009). Moreover, IL-10 inhibits class switching to IgE (Punnonen et al. 1993) at the level of germ line transcripts. This effect was shown to be dependent on the presence of monocytes in the culture system and was independent of inhibition of other cytokines, including IL-6 and TNF-α (Punnonen et al. 1993).

4.3 DiseaseS associated with deficiency of IL-10

IL-10 deficiency in mice results in resistance to infection especially chronic viral infections (Ejrnaes et al. 2006; Brooks et al. 2006). IL-10 deficient mice exhibit efficient clearance of chronic virus infection, which results from enhanced action of effector Th1 cells and IFN-γ producing CD8+ T cells (Ejrnaes et al. 2006; Brooks et

al. 2006). On the other hand, IL-10 deficient mice are also more susceptible to immunopathology from exaggerated Th1 responses (IL-12, TNF-α and IFN-γ) after certain infections, including toxoplasmosis and plasmodium (Gazzinelli et al. 1996; Li, Corraliza, and Langhorne 1999). In fact, exaggerated and uncontrolled Th1 responses can be triggered even by gut bacterial commensals (Kuhn et al. 1993; Takeda et al. 1999). In humans, genetically determined IL-10 or IL10R deficiency results in very early-onset and severe colitis (Kuhn et al. 1993; Berg et al. 1996; Sato et al. 2006; Engelhardt et al. 2013; Engelhardt and Grimbacher 2014) and (Glocker et al. 2010; Kotlarz et al. 2012; Glocker et al. 2009; Lee et al. 2014). Aside from these rare damaging mutations, certain more common *IL10* polymorphisms confer an increased risk of IBD, especially ulcerative colitis (Franke et al. 2008).

5. T cell exhaustion

5.1 IL-10 and T cell exhaustion

IL-10 deficiency renders humans more susceptible to inflammatory diseases. On the other hand, up regulation of IL-10 by APC or by CD4+ T cells (Ejrnaes et al. 2006; Brooks et al. 2006; Wherry 2011) during the course of viral infections is associated with the risk of chronic and persistent infection (Brooks et al. 2006). Conversely, IL-10 inhibition has been shown to enhance control of virus replication through restoration of the effector T cell functions (Brooks et al. 2006; Porichis et al. 2014). This is mainly due to the inhibitory action of IL-10 on effector Th1 CD4+ and CD8+ T cells. These cells lose the ability to proliferate and produce their inflammatory cytokines such as (IL-2, IFN-γ and TNF-α) (Brooks et al. 2006; Zajac et al. 1998). This state is called T-cell exhaustion (Yi, Cox, and Zajac 2010; D'Souza et al. 2007; Wherry 2011).

5.2 PD-1 and T cell exhaustion

High level antigenic stimulation, as occurs during chronic viral infections, drives T cell exhaustion, a state associated with impaired effector T cell responses (Mueller and Ahmed 2009). T cell exhaustion is very well-described process in CD8+ T cells

(Yi, Cox, and Zajac 2010) (and reviewed by Akbar, A.N.(Akbar and Henson 2011) and Wherry, E.J.(Wherry and Kurachi 2015)). PD-1 is an established marker of clonal exhaustion on CD8+ T cells (Akbar and Henson 2011; Buggert et al. 2014; Wherry and Kurachi 2015) in chronic viral infections such as HIV (Day et al. 2006; Buggert et al. 2014), and chronic hepatitis virus infection (Ye et al. 2015). Expression of PD-1 by exhausted CD8+ T cells marks them as highly sensitive to spontaneous apoptosis and Fas-induced cell death, (Petrovas et al. 2009), which results in progressive loss of antigen specific T cells (Yi, Cox, and Zajac 2010). High expressing PD-1 ($PD\text{-}1^{hi}$) exhausted cells are characterized by low levels of T-bet expression but high levels of Eomes (Buggert et al. 2014). Blockade of PD-1 has been shown to reverse aspects of the exhaustion phenotype in CD8+ T cells (Day et al. 2006). While better characterised in CD8+ T cells, clonal exhaustion of CD4+ T cells has been described, and is also characterised by up regulation of inhibitory molecules such as PD-1 (Morou, Palmer, and Kaufmann 2014; Day et al. 2006). Furthermore, in CD4+ T cells, blocking PD-1 liberates them from their nonresponsive state and promotes production of inflammatory cytokines such as IFN-γ and IL-2, and restores capacity for cell proliferation (Rosignoli et al. 2009; Porichis et al. 2011; Porichis et al. 2014).

5.3 T cell senescence

Chronic antigenic stimulation of terminally differentiated effector CD8+ T cells can also induce cellular senescence, in which T cells can retain effector and cytotoxic capacity, but lose their replicative potential. This is in contrast to the exhausted cell, where cells have impaired proliferative capacity coupled with impaired functional abilities and reduced cytotoxicity (Akbar and Henson 2011). CD57 is thought to be a specific marker of senescence (Larbi and Fulop 2014). Senescent cells are characterized by short telomeres (Akbar and Henson 2011; Larbi and Fulop 2014; Moro-Garcia, Alonso-Arias, and Lopez-Larrea 2012).

5.3.1 CD57

CD57 was initially thought to be a marker of natural killer (NK) cells only (Abo and Balch 1981) but was later found to be expressed by other lymphocytes. As noted above, in general, CD57 marks senescent terminal effector cells with reduced proliferative capacity (Kared et al. 2016). CD57 is expressed by mature CD56 ^{dim} CD16 +NK cells (Abo, Cooper, and Balch 1982), and expression of CD57 by NK cells increases naturally with age (Lopez-Verges et al. 2010), but this process appears to be accelerated in the setting of chronic antigenic stimulation, such as autoimmune diseases, chronic viral infection, and cancer (Nielsen et al. 2013). In NK cells, CD57 is a marker of cytolytic activity with enrichment for granzyme-A, granzyme-B and perforin (Chattopadhyay et al. 2009). Increased or enhanced cytolytic function of CD57+ NK cells was found to be associated with better outcome in different malignancies in support of their function in tumour surveillance (Nielsen et al. 2013). CD57+CD8+ T cells are also increased in setting of antigenic stimulation such as in HIV and CMV (Bandres et al. 2000; Brenchley et al. 2003; Akbar and Henson 2011; Petrovas et al. 2009; Strioga, Pasukoniene, and Characiejus 2011; Le Priol et al. 2006). They are highly effector, cytotoxic and distended for homing to peripheral tissues (Le Priol et al. 2006).

5.3.1.1 CD57+ CD4+ T cells

CD57 is mainly expressed by peripheral terminal effector CD4+ T cells, both Temra and Tem subsets in humans (Palmer et al. 2005; Velardi, Grossi, and Cooper 1985; Di Mitri et al. 2011). The CD57 +CD4+ T cells subset has been found to be expanded in states of chronic immune activation (Fleischer 1984), including in patients with EBV or HIV infection (Palmer et al. 2005), autoimmune diseases such as rheumatoid arthritis (Maeda et al. 2002; Appay 2004), and IgG4 related disease (Mattoo et al. 2016). CD57+ CD4+ T cells produce mainly IFN-γ (Palmer et al. 2005). Similar to CD8+ T cells, expression of CD57 by CD4+ T cells identifies cellular senescence characterised by proliferative incompetence (Palmer et al. 2005; Brenchley et al. 2003; Di Mitri et al. 2011).

Recent reports have also shown that human peripheral blood CD57+ CD4+ are terminally differentiated effector but also possess cytolytic potential marked by expression of granzyme-A, granzyme-B , perforin and CD107a degranulation (Casazza et al. 2006; Chattopadhyay et al. 2009). In addition to circulating CD57+ CD4+ T cells, there is also a subpopulation of CD57+ CD4+ T cells that reside in the tonsil as TFH cells (Sattarzadeh et al. 2015; Gomez-Martin et al. 2011; Bouzahzah et al. 1995). It remains unclear whether this TFH subset is functionally distinct, with conflicting evidence regarding their capacity to help B cell differentiation, relative to their CD57- counterparts. CD57+ TFH cells are thought to lack cytolytic activity based on analysis of their ability to kill K562 cell lines. (Velardi, Grossi, and Cooper 1985). .

6. Summary of literature and points to be discussed

6.1 Human T cell differentiation by STAT4

The capacity of peripheral T cells to differentiate into different effctor subsets is a result of interplay between TCR ligation, cytokine stimulation, co-stimulation and or co-inhibition signals. The three stimuli converge on specific transcription factors that mediate the signal to switch on or switch off certain transcriptional regulators that result in adoption of specific T cell fate.

In animal models, it has been shown very clearly that STAT4 is needed for Th1 T cell formation, and is associated with T-bet up-regulation and IFN-γ production. STAT4 has also been implicated in the formation of TFH and cTFH based on association studies in patients with IL-12Rβ1 deficiency, together with detection of STAT4 in normal germinal centres. Despite the suggestions from previous reports, there is as yet no conclusive genetic or functional evidence for *STAT4* gain of function or loss of function mutations in humans. This has impeded any confirmation what has been suggested from the animal models and *in-vitro* assays in regard to contribution of human STAT4 toward formation of human Th1, cTFH and TFH.

6.2 MTOR in human T cell differentiation

Different types of effector CD4+ and CD8+ T cells can also be classified according to expression of CCR7 and CD45RA. Of the many molecules important in guiding T cell differentiation from naïve to different effector and memory subsets, mTOR has been elegantly shown to be required for effector Tem development in animal models especially for the CD8+ T cell subset. The Temra subset is lacking in mice, and therefore can only be investigated in humans. Human mutations in PI3K have been shown to result in enhanced mTOR activity, and are associated with deviation toward terminally differentiated (Tem and Temra) effector CD4 and CD8+ T cells with a Th1 phenotype (Angulo et al. 2013; Lucas et al. 2014). Finally, mouse studies have also shown that mTOR can enhance STAT4 signalling and Th1 deviation in the CD8+ T cell compartment. There are no human 'models' available to confirm the action of mTOR in the differentiation of CD8+ T cell subsets, especially Temra, and the cross regulation of STAT4 and mTOR.

6.3 STAT regulation of IL-10 and T cell exhaustion

STAT3 loss of function (LoF) mutations are now well-characterised syndrome that results in Th17 deficiency in humans. The pathophysiology of the high level of IgE in HIES, however, remains enigmatic. The level of IgE does not become high until patients reach 1 year of age(Kamei and Honig 1988). Interleukin- 4 (IL-4) response as a main Th2 cytokine is a well know inducer of class switching to IgE (Romagnani 1994; Avery et al. 2008). Unopposed IL4 has been suggested to cause the high IgE in ADHIES. However, this simple Th2 bias is not a consistent finding in peripheral blood in patients with HIES. IL-10 produced by CD4+ Th2 cells has also been shown to control excessive Th2 and IL-4 immunopathology in animal models. Formation of this regulatory subset was shown to be enhanced by STAT3-dependent cytokines, pointing to the possibility that a defect in this IL-10 producing subset might contribute to Th2 immunopathology seen in *STAT3* LoF.

TFH are distinct subset of helper CD4+T cells that are efficient in supporting B cell differentiation, plasma cell formation and immunoglobulin class switching and

production. TFH and their blood counterpart cTFH cells are commonly identified by their surface markers such as high and intermediate level of CXCR5 respectively. In addition, TFH cells also express other markers such as PD-1(exhaustion marker) with or without CD57 (cytotoxic marker) among other markers. Formation and function of TFH and cTFH are comprised in patients with AD-HIES (*STAT3* LoF) defined by the classical marker of CXCR5. However, it is not clear if there is a clear cytotoxic or exhaustion distinction between the two subsets based on expression of CD57 by TFH and cTFH. Moreover, there is no data to implicate STAT3 in the formation of these two subsets.

In this thesis, we describe the results of experiments aimed at investigating the actions of STAT4 and STAT3 in controlling specific aspects of T cell differentiation. First, we describe analysis of immunity in a patient with a novel *STAT4* gain of function (GoF) mutation. We confirm the importance of human STAT4 for Th1 differentiation namely, T-bet up regulation, IFN-γ production and we show that STAT4 is crucial for cTFH formation *in vivo*. We also report a gain of function mutation in *MTOR* and demonstrate the consequences for effector CD4+and CD8+ T cell differentiation, as well as cross regulation between STAT4 and mTOR during Th1 and TFH differentiation.

Finally, using cells from patients with functional mutations in *STAT3,* we have characterised how STAT3 regulates IL-10 producing Th2 cells, T cell exhaustion, and acquisition of CD4+ T cell cytotoxicity by CD57+ CD4+ T cells.

CHAPTER 2 : MATERIALS AND METHODS

1 Subjects

Data and samples of patients and controls were collected as a part of the Australian and New Zealand Antibody deficiency allele study (ANZADA). The collection of blood and saliva was according to a standard operating procedure. Tissue donated at time of diagnostic biopsy as part of patient's management plan after obtaining an informed consent.

2.1.1 Ethical approval and informed consent

All of the detailed work on samples of patients and controls (healthy volunteers) described and used in this thesis have been approved by local human research ethics committee at each institution. Patients and controls were given information sheets and verbal explanation prior to obtaining a signed consent form. All patients' information with identifications were coded and kept securely.

2.1.2 Subjects with *STAT3, STAT4, MTOR* mutations

Subjects with *STAT3* mutations were genotyped previously and the work has been published (Ma et al. 2008). Mutations in the initial subject with GoF-*STAT4* and GoF-*MTOR* were identified from the whole exome sequencing as part of this discovery project. These mutations were then confirmed in the same subjects and other available family members using Sanger sequencing.

2 Statistical analysis

The results were analysed with MedCalc software (Mariakerke, Belgium) and Microsoft Excel (Washington, USA). The differences between paired samples were

analysed using either independent student T test, Mann Whiteny or Wilcoxon rank test (for non-parametric paired samples).$P<0.05$ is considered statically significant.

3 Materials and Methods

2.3.1 Material and reagents

All of the material used for the work of this thesis are listed in the below given tables from 2-1 to 2-11.

Table2-1 Media and buffers for the cellular work.

Media/ Buffer	Ingredient	Final concentration	Source
Culture Media (CM)	RPMI 1640 medium		Sigma Aldrich
	Fetal calf serum(FCS)	10%	Life Technologies
	L-glutamine200 mM	4mM	Sigma Aldrich
	Pencillin10000U/ 10mg/ml) Streptomycin	100U/ml /100 µg /ml	Sigma Aldrich
Freezing Media(FM)	CM as above		
	Dimethyl sulfoxide (Hybri Max)(DMSO)	10%	Sigma Aldrich
FACS wash	PBS		Gibco
	FCS	2%	Life technologies
Sterile FACS wash	PBS		Gibco
	FCS	0.1-0.2%	Life technologies
Perm wash buffer 1x (cytokine assays)	BD Perm wash buffer 10X	1X	BD
	Distilled water,9 parts		
Fix buffer (FoxP3)	FoxP3 Buffer A x 10, 1 part Distilled water ,9 parts	1X	BD
Perm buffer(Foxp3)	FoxP3 Buffer B (50X), 1 part	1X	BD

Table 2-2 Various cytokines and stimulants and their doses

Name	Source	Final concentration
Phytohemagglutinin(PHA)	Sigma	25 µg /ml
T cell activation and expansion beads	Miltenyi Biotech	2:1 beads to cell
Phorbol 12-myristate 13-acetate (PMA)	Sigma	50nM
IFN-α	Schering-Plough	20IU/ml
Recombinant human IL-1β	Miltenyi Biotec	40ng/ml
Recombinant human IL-2	Thermo-Fischer scientific	80ng/ml
Recombinant Human IL-4	R&D	50ng/ml
Recombinant Human anti- IL-4	R&D	20 µg /ml
Recombinant human IL-6	Miltenyi Biotec	200ng/ml
Recombinant human IL-10	R&D	100ng/ml
Recombinant Human IL-12	R&D	20 ng/ml
Recombinant Human IL-21	Mitenyi Biotec	10ng/ml or100ng/ml
Recombinant Human IFN-γ	PeproTech	
Recombinant Human IFN-γ neutralizing	BD	10 µg /ml
Recombinant Human sCD40 Ligand	PeproTech	1 µg /ml
Type B CpG oligonucleotide human TLR9 ligand(ODN2006)	Invivogen	1uM
Rapamycin	sigma Aldrich	10ng/ml, or 100 ng/ml
Anti Igs [(Fab)2 (] Goat anti	Jackson ImmunoResearch	5 µg /ml

human IgG,F(ab)2 fragment specific)	Laboratories	

Table 2-3 Reagents for molecular and western blot work

Reagent	Company	Catalogue number
Trizol	Invitrogen	15596
Chloroform	Sigma-Aldrich	
100 % Isopropyl alcohol		
75% ethanol (in DEPC-treated water)		
miScript Reverse Transcriptase mix. miScript RT Buffer 5 x	Qiagen	218161
Phusion Hot Start II High-Fidelity DNA Polymerase2U/ul. 5X Phusion HF Buffer. Phusion Hot StartII.	Thermo Scientific	F-549S
10mM dNTPs		
Subcloning Efficiency™ DH5α™ Competent Cells	Invitrogen	18265-017
PcDNA™3.1/myc-His (-) A plasmid	Invitrogen	V855-20
QIAprep Spin Miniprep Kit	QIAGEN	27106
GelRed Nucleic Acid Stain10,000x	Biotium	41003
Gel loading dye blue 6x	New England Biolabs	
1Kb Plus DNA ladder	Invitrogen	10787-018
Wizard® SV Gel and PCR Clean-Up System	Promega	A9282
KpnI restriction enzyme 10,000 unit/ml with 1X NEBuffer 3.1	New England Biolabs	R0142
BamHI restriction enzyme 20,000	New England Biolabs	R0136

unit/ml with 1X NEBuffer 3.1		
T4 DNA ligase 3 Weiss unit/ul	New England Biolabs	M0202S
10x T4 DNA Ligase Reaction Buffer		
Nunc EasYFlask 175cm2	Thermo Scientific	159910
2.5% Trypsin 10x	Gibco	15092
lipofectamine LTX and PLUS reagent kit	Invitrogen	15338
STAT4 luciferase reporter vector	Millennium Science Pty Ltd	PAN LR0079
Sterile 96 well solid white flat bottom polystyrene TC-treated microplates	Corning	3917
Dual-Glo® Luciferase Assay System	Promega	E2920
lipofectamine LTX and PLUS reagent	Invitrogen	15338
Halt Phosphatase Inhibitor Cocktail, 100X	Pierce	78420
Protease Inhibitor Cocktail Set III, EDTA-Free	Calbiochem	539134
RIPA	Sigma	r0278-50ml
True Sep SDS sample buffer		BG-145
Tween 20	Sigma,	P2287
Tris glycine mini gel 10 and 12 %	nusep	NB10-010& NB10-012
Tris -Glycin SDS Running Buffer (10x)	life technologies	Lc2675
Transfer Buffer 20X	lifetechnologies	NP00060
Immun-Blot PVDF Membrane	BioRad	162-0177
Albumin from bovine serum	Sigma	A7906-100g
Amersham ECL Western Blotting Detection Reagents	GE Healthcare A	RPN2106

Table2-4 The master mix for generation of cDNA

Reagents	volume
5x miScript RT Buffer	4μL
miScript Reverse Transcriptase mix	1μL
Template RNA	Volume making up 1μg
RNase-free water	To make up total of 20ul

Table2-5 Amplification of *STAT4*

Reagent	volume
5X Phusion HF Buffer	10 μL
10mM dNTPs	1μL
Primer forward ggctccaggatccaccatgtctcagtggaatcaag	2.5ul(10pmol)
Primer reversed gg ggt acc ttc agc aga ataaggagacttca	2.5ul(10pmol)
cDNA	1ul
Phusion Hot StartII	0.5ul
RNase-free water	32.5 ul

Table2-6 Media and Buffers for the molecular and western blot work

Name	Reagent	Volume	Notes
	10g of tryptane	1 L H2O	PH of 7.0 sterilized in autoclave at 121°C for 15 minutes and left in 4°C
	5g of yeast extract		
	5 g of NaCl2		
LB –amp media	LB-media		for short time use
	fresh ampicillin at a final of 100 μg /ml		
LB-amp	The LB media		Autoclaved at

agar plate	500 ml (pH adjusted to 7.5)		121°C, 15mns. Cool down to around 50C. Plates poured. left overnight
	7.5 g of agar		
	fresh ampicillin at a final concentration of 100 µg /ml		
TAE buffer 50x	242g Tris base	1 L	
	57.1ml Glacial Acetic Acid		
	18.6gEDTA		
	900ml of distilled H_2O		
1% agarose gel	1 gram of agarose		boiled in a microwave for 1-2 minutes till all components are well dissolved
	100 ml of (1x) TAE buffer		
	10ul of GelRed Nucleic Acid Stain		added to the cool mixture
Cracking buffer	5mM EDTA		
	50mM NaOH		
	0.5% SDS		
Full media of DMEM	High glucose DMEM (Gibco)		
Transfection media	DMEM		
	4mML-glutamine		
Plating media	High glucose DMEM435 ml	Gibco (11960-069)	
	FCS 50 ml	Invitrogen(16140-071)	
	L-glutamine200mM(5ml)	Sigma(59202c)	
	Pencillin10000U/ 10mg/ml) Streptomycin(5ml)	Sigma (p4333)	
Working RIPA buffer	diluting the 10x in distilled water	Prepared and used on the same day	

	1x Protease Inhibitor Cocktail, 1x Halt Phosphatase Inhibitor Cocktail	
Working Cytobuster buffer	Cytobuster buffer 1x Protease Inhibitor Cocktail 1x Protease Inhibitor Cocktail	Prepared and used on the same day
10X Tris Buffered Saline (TBS). 2L	121 g Tris base 15 g NaCl; adjust pH to 7.6 with HCl ~70ml (use at 1X)	
Chilled transfer buffer2 L	50ml of transfer buffer 100 ml of methanol 850 ml of water	Mixed and left at -20 C ~2 hours before use
Wash buffer (TBS/T).	1xTBS 0.1%Tween-20	
Working SDS	10% mercaptonethanol SDS buffer	

Table 2-7 Sequence analysis of *STAT4* for Sanger sequencing

STAT4 vector F1	CCAACATGCCTGTCCAGGGGC
STAT4 vector F2	GGAAGCGGCGGCAGCAAATC
STAT4 vector F3	GGGCTGTCACATGGTGACTGAAG
STAT4 vector F4	GGTGGACCATTCTGAAAGTGGGG
STAT4 vector R3	GCCAGCTCATCACCTCCAGT

Table2-8 Antibodies used for the western blot work

Antibody	Clone	Source	Dilution
Rabbit anti-Phospho-p70 S6 Kinase; Thr389	108D2	Cell Signaling Technology	1/100
Rabbit anti- P-MTOR(Ser2448)	(D9C2) XP	Cell Signaling Technology	1/500
Mouse anti-STAT4(PY693)	38/p-Stat4	BD	1/200
Rabbit anti STAT4	polyclonal	Invtrogen	1/100
Goat anti-mouse IgG-HRP	polyclonal	Santa Cruz	1/4000
Sheep anti- rabbit IgG	polyclonal	Abcam	1/4000
Mouse anti GAPDH-100 µg	6C5	Abcam	1/5000
Mouse anti -TATA binding	1TBP18]	Abcam	1/5000

2.3.2 Methods

2.3.2.1 Separation and freezing of peripheral mononuclear cells (PBMCs) from blood

Freshly drawn blood in anticoagulant tubes such as ACD tubes, were mixed by inverting the tube several times before diluting it 2:1 Blood: PBS in 50 ml falcon tube. After this, tubes were mixed by inverting the tube several times. The blood was poured directly into the leucosep tube (containing ficol under the filter) and centrifuged at 1000g for 15 minutes, no break, at ~20°C. The buffy coat was then aspirated using a sterile pipette and transferred into new falcon tubes and the sample was washed 2-3 times with PBS or cold RPMI at 350g for ~5-8 minutes with breaks on. The cells were counted using trypan blue staining before the last wash. The supernatant of the last wash

was discarded and the cell pellet was dislodged using gentle quick vortex and a freshly thawed freezing media was added to the dispersed cell pellet to make a final concentration of ~2millions of cells /1 ml of the freezing media. The sample was aliquoted into pre-labelled Nunc freezing vials which were then placed into Isopropanol container and then into -80°C freezer overnight. The samples were then moved into liquid nitrogen for long term storage.

2.3.2.2 Separation and freezing of peripheral mononuclear cells (PBMCs) from tonsil

Fresh extracted tonsils from individuals undergoing routine tonsillectomy was kept in culture media (CM) till processing, which was usually performed within 1-2 hours post-tonsillectomy. The tissue was put inside large plastic Petri dish containing either cold Roswell Park Memorial Institute medium (RPMI) or CM. The tissue was then cut into 3- to 10-mm fragments with scissors or scalpels. The cell suspension was then subjected to separation by Ficoll as described above. The cells were washed twice and before the last wash, counted by tryptan blue. The tonsil cells were frozen at ~10 million/ml (Johnston, Sigurdardottir, and Ryon 2009).

2.3.2.3 Cell Thawing

The desired number of the vials were taken out of the freezer and placed on ice. Thawing of the samples was done by thawing individual vial at 37°C for1-2 minutes, wiping the vial with ethanol to prevent contamination, adding the sample into a pre-labelled falcon tube half filled with warm CM. The sample was then centrifuged at 350*g* for ~5-8 minutes with breaks on. Next, the supernatant was decanted, the pellet dislodged by gentle flicking and fresh media was added for another wash. The sample was counted just before the third wash and re-suspended as desired for the assay. Proceed with the required assay as per protocol.

2.3.2.4 Ribonucleic acid (RNA) isolation and purification

In a biological safety cabinet dedicated to ribonucleic acid (RNA) work, RNA extraction was done on PBMCs following the company protocol. Briefly, to homogenise the sample, 0.5ml Trizol was added to a pelleted lymphocytes (~<1 x 10^6 cells) in a 15 ml flacon tube, mixed by pipetting up and down several times and incubated for 5 minutes at room temperature(RT). The cells were then transferred into 1.5 ml microcentrifuge tube. The homogenised sample was separated by adding 0.1ml of chloroform after which the tube was capped and subjected to vigorous shaking for 15 seconds and incubation at RT for ~3 minutes. The sample was then centrifuged at 12,000*g* for 15 minutes at 4°C. The result of this was 3 phases in the tube. The colourless upper aqueous phase contained RNA which was transferred carefully into a new microcentrifuge tube. 250ul of 100% isopropranolol was added, followed by incubation at RT for 10 minutes for RNA precipitation. The RNA was pelleted at 12,000g for 10 minutes at 4 °C. The supernatant was removed carefully by pipetting. The RNA pellet was washed using 500 ul of 75% ethanol (in DEPC treated water), this was followed by a brief vortex followed by centrifugation at 7500 x g, 4°C, 5 minutes. The ethanol was decanted and the RNA pellet was left to air dry for 5-10 minutes to avoid over drying and compromising the quality of the pellet. The pellet was rehydrated in ~20-30ul of RNase–free water via gentle pipetting. The resultant RNA was incubated in a heat block at 55-60°C for 10-15 minutes. The yield of the RNA sample was determined at absorbance of 260/280nm, left on ice till preparing the reaction for the cDNA conversion.

2.3.2.5 Reverse transcription

The template RNA was either frozen at -20°C or used immediately, in which case was left on ice while preparing the rest of the reaction. miScript RT buffer,5x and RNase-free water were removed from the kit and left to thaw at RT (15-25°C), the tubes were flicked and centrifuged briefly to mix their content. The miScript Reverse Transcriptase mix was removed from the freezer and left on ice just when everything was ready to be

added into the master mix. The master mix was prepared following the company protocol for RNA (10pg-1 µg) as below in a total volume of 20ul.

The template RNA was added last and mixed. The tube containing the mixture was incubated for 60 min at 37°C followed by 95°C, 5 minutes and last was left on ice for ~5-10 minutes. The newly synthesized cDNA was placed in a -20°C freezer till further use for *STAT4* amplification.

2.3.2.6 Amplification of STAT4 gene

Primer forward ggctccaggatccaccatgtctcagtggaatcaag,

Primer reversed gg ggt acc ttc agc aga ataaggagacttca

A master mix was prepared for the reaction in a total volume of 50 ul in a 0.2ml thin-walled polypropylene PCR tubes as follows:

The reaction mix was mixed by pipetting and subjected to quick spin and placed in a thermo cycler under the following conditions.

Table 2-9 Conditions for *STAT4* amplification

Step	Temperature	Duration	Cycle
Initial denaturation	98°C	30second	1
Denaturation	98°C	10second	2-30
Annealing	55°C	30second	2-30
Extension	72°C	2.5minutes	2-30

The PCR product was stored at -20°C till subsequent use for electrophoresis.

2.3.2.7 Transformation to be cloned into pcDNA™3.1/myc-His (-) A vector, growing pcDNA™3.1/myc-His (-) A plasmid

25 ul of aliquoted Sub cloning Efficiency™ DH5α competent cells in a microcentrifuge tube was removed from -80°C and placed directly on ice for 5 minutes to thaw, the cells were gently mixed by tapping the tube, and 0.5 ul (25ng)of

pcDNA™3.1/myc-His (-) A plasmid was added aseptically. The tube was flicked gently and placed then on ice for 30 minutes. The tube was then put in a 42°C heat block for 45 second after which the tube was placed on ice immediately. 225ul of RT LB media was added to the tube, which was then incubated for ~1hour at 37°C with shaking at 225 rpm. 100 ul of the reaction was plated onto LB-amp agar plates using inoculation loop. 2 plates were prepared. The plates were then incubated overnight (~16) hours at 37°C. The next day, single colonies were picked up aseptically and inoculated into a 10 ml LB/amp media and left to grow at 37°C on a shaker overnight (~16).

QIA spin Miniprep Kit was used to isolate the vectors following the manufacture protocol. Briefly, the culture was pelleted by centrifugation at maximum speed of 13000rpm for ~5 minutes, the supernatant was decanted, and the manufacture lysing buffer was added to the pellet which was then transferred into a new microcentrifuge tube pellet. The lysate was cleared by centrifugation. This clear lysate was then applied to a column to bind, followed by a washing step to purify the plasmid DNA. The pure DNA was eluted in small volume (~50ul/tube) of water after which the concentration was measured and the sample was stored at -20°C till further use.

2.3.2.8 Nucleic acid electrophoresis

Gels were run in TAE buffer. 10 ul of working loading ladder (5µL of 1Kb plus DNA ladder mixed with 1µL loading dye) was added to the first well. The rest of the samples were loaded to the remaining wells and electrophoresis was performed at 100V of ~1.5hour. The gel was then visualised under UV light. For isolation of amplicons, band were excised in a minimum volume of agarose using disposable sterile scalpels, weighed and purified using membrane based system following the company protocol to eliminate the residual nucleotides and primers. Briefly the gel slice was dissolved using the buffer provided, applied to the provided column and the DNA recovered using a centrifugation system. Then, the recovered DNA was washed

using the provided wash solution and then eluted in water. Finally the concentration was determined and stored at -20°C till further use.

2.3.2.9 Digestion and of the STAT4 product and the pcDNA™3.1/myc-His(-) A vector

A sequential digest was performed on the *STAT4* product as well as the pcDNA™3.1/myc-His (-) A, following the below tables. The sequential digest was done due to incompatibility of the two chosen enzymes and their optimal buffers.

Table 2-10 Conditions for *STAT4* digestionI

Reagents	volume
DNA	volume making up 1 µg ~
Buffer I	2.5ul
KpnI	1ul
BSA	0.25ul
RNase-free water	To make up total of 25ul

The reaction was mixed by pipetting, followed by a quick (touch) spin-down and NO Vortex. The reaction mix was then incubated in a heat block 37°C for 2-3 hours. After which the following was added to the same mix.

Table 2 Conditions for *STAT4* digestion II

Reagents	volume
BufferIII	2.5ul
BamHI	0.5ul
BSA	0.25ul
RNase-free water	To make a total of 50 ul

Once again, the reaction was mixed by pipetting, followed by a quick (touch) spin-down and NO Vortex. The reaction mix was then incubated in a heat block 37°C for

another 2-3 hours. The reaction mix was then stored at -20°C for further use such as DNA electrophoreses (especially for the vector to make sure of the efficiency of the digestion step).

2.3.2.10 Ligation of the STAT4 product and the pcDNA™3.1/myc-His(-) A vector

The appropriate amount of the PCR, vector to be added was determined using the below formula to give the optimal molar ratio of PCR product: vector (6:1).
Digested pcDNA™3.1/myc-His (-) A= 45ng/ul
PcDNA™3.1/myc-His (-) A=5.5kbp
Digested *STAT4* PCR product= 21.6 ng/ul
STAT4 is 2.2kbp

$$\text{Insert mass (ng)} = \frac{6 \times \text{insert length (bp)}}{\text{Vector length (bp)}} \times \text{vector mass (ng)}$$

Table 2-11 Ligation of *STAT4* with its vector

Reagents	volume
Digested pcDNA™3.1/myc-His (-) A	1 ul
Digested *STAT4* PCR product	5ul
10x T4 DNA Ligase Reaction Buffer	2ul
T4 DNA ligase	1ul
RNase-free water	11ul (a total of 20ul)

The reaction was set up at the end of the day left to continue over night at RT on the bench.

2.3.2.11 Transformation

The next day a transformation reaction was performed using 50 ul of aliquoted MAX Efficiency® DH5α™ Competent Cells in a microcentrifuge tube. The MAX Efficiency® DH5α™ Competent Cells were removed from -80°C and placed directly on ice for 5 minutes to thaw, the cells were gently mixed by tapping the tube, and 10 ul of the ligation product was added aseptically. The tubes were flicked gently and placed then on ice for 30 minutes. The tube was then put in a 42°C heat block for 45 second after which the tube was placed on ice immediately. 450ul of RT LB media was added to the tube, which was then incubated for ~1hour at 37°C with shaking at 225 rpm. 100 ul of the reaction was plated onto LB-amp agar plates using inoculation loop. The plate was then incubated overnight (~16) hours at 37°C. By the next day the cultures have turned very cloudy. Single colonies were picked up and inoculated into a 2 ml LB/ampicllin media and incubated at 37°C overnight with a shake. The next day QIA spin Miniprep Kit was used to isolate the vectors following the manufacture protocol as briefed above.

2.3.2.12 Isolating colonies with STAT4 insert

The cracking method was used to identify the colonies with *STAT4* insert. ~250 ul of the bacterial cultures were aliquoted into microcentrifuge tube which was then centrifuged using maximum speed for 1 -2 minutes to pellet the bacteria. The supernatant was decanted. 20-30 ul of the cracking buffer was added to the bacterial pellet, vortexed and incubated at 65°C for 15 minutes to lyse the bacteria and release both the genomic as well as the plasmid DNA. After which the samples exhibited high viscosity, vigorously vortexed for 1 minute, followed by quick centrifuge to pellet the sample and carefully loaded using a wide tip pipette into 1% agarose gel (with wider lane to accommodate larger volume) and electrophoresed for 2-3 hours at 100V. The time was judged by seeing maximum separation of the control dye. The gel was viewed under UV-illuminator and the colonies corresponding to ~6 kb (had

STAT4 insert) were grown in 10 ml culture, followed by plasmid extraction and DNA quantification which were done as described above.

2.3.2.13 Sequence analysis

Primers listed in table 2-12

Plasmids extracted by the QIA spin Miniprep Kit and were sent for Sanger sequencing using the listed primers to identify the normal from the mutant allele.

2.3.2.14 Maintenance of HEK293T

HEK293T were maintained in DMEM until ~90-95% confluent, and then passaged by aspirating the old media gently, rinsing in PBS once and then adding 2 ml of (1x) trypsin, swirling the flask and putting it back into the 37°C incubator for 5 minutes, after which the cells had detached. This was followed by washing in fresh full warm media, centrifugation at 350g for 5 minutes and then re-suspended in growth media in 1:10 ration for a maintenance purpose.

2.3.2.15 Transfection for Luciferase

HEK293T cells which were maintained in full media were harvested by trypsin treatment as described above. Cells were washed, re-suspended at concentration of ~2 x $10^{5/}$ ml of plating media. 500 ul /well of 24 wells plate was plated aiming for ~ 80-90 % at the time of transfection. Plasmid DNA; 0.2 µg per well of the either the wild or mutant *STAT4* alleles, 0.2 µg of the Stat4 Luciferase Reporter Vector, 0.2 µg of pRL-CMV Vector and 0.2 µg of PcDNA™3.1/myc-His (-) A making up a total of 0.8 µg of plasmid DNA which was used for cell transfection with the lipofectamine LTX and PLUS reagent kit following the company protocol. Briefly, a master mix tube was prepared for both the wild type and the mutant according to how many transfections of 24 well were needed following the table below. In this experiment each transfection was done in triplicate. The transfection media was added to a labelled microcentrifuge tube, followed by adding the calculated volume of the

plasmid, the mixture was mixed by pipetting, and then the plus reagent was added. The mixture was then mixed gently by pipetting. The mixture was incubated for 5 minutes RT. The lipofectamine LTX was added separately to tubes with transfection media which was then added to the mixture containing the DNA and plus reagent and then incubated for further 30 minutes at RT. The whole final mixture was then added to the plated HEK293T in a drop wise manner and the plate was rocked gently and put back again in 37°C CO2 humidified incubator.

STAT4 Plasmid (0.2 µg)	volume making up	0.2 µg	x3 wells
IRF1	volume making up	0.2 µg	x3 wells
pRL-CMV	volume making up	0.2 µg	x3 wells
PcDNA™3.1/myc-His (-) A	volume making up	0.2 µg	x3 wells
Plus	xul (=1* DNA µg)=0.8ul		x3 wells
DMEM/L-glutamine	50ul		x3 wells
The mixture was mixed and incubated for 5 minutes			
Lipo	xul= (2.5*DNA µg)=2ul		x3 wells
DMEM/L-glutamine	50ul		x3 wells

The latter mixture was mixed with the former mixture and incubated for 30 minutes.

The cells were inspected for media consumption and old media was replaced at 16-18 hours post transfection. Cells were then counted and ~ $6x10^4$ / well were transferred into 96 white flat bottom polystyrene plate and then left for a total of ~36- 40 hours post transfection to express the protein before stimulation with IFN-α (20IU/ml vs 3000IU/ml) for 6 hrs, vs 24 hours. The activation was terminated at the given time by washing the cells twice with RT, 1X PBS. The cells were subjected to the luciferase assay following the company protocol (Dual-Glo Luciferase Assay System). Briefly, the cells were resuspended in 50 ul of RT PBS /well. 50 ul of Dual-Glo luciferase reagent was added /well and mixed by pipetting. The plate was incubated for ~15 minutes RT dark after which the firefly signal was read by a luminometer. 50 ul of Dual-Glo Stop & Glo Reagent /well was added immediately and the plate was then incubated RT dark for ~20 minutes and the renilla signal was read by luminometer.

The signal of the firefly of the sample was then normalized against the signal of the renilla of the same sample.

2.3.2.16 Transfection for western blot

HEK293T cells which were maintained in full media of were harvested at ~90% confluency by trypsin treatment as explained above. The cells were washed, re-suspended at concentration of ~2 x $10^{5/}$ ml of plating media and 500 ul /well of 24 wells plate was plated aiming for in 70-90 % at the time of transfection . Plasmid DNA (0.8 µg per well) carrying either the wild or mutant *STAT4* alleles was used for cell transfection with the lipofectamine LTX and PLUS reagent kit following the company protocol. Briefly a master mix tube was prepared for both the wild type and the mutant according to how many transfections of 24 well are needed following the table below. The plating media was added to a labelled micro-centrifuge tube, followed by adding the calculated volume of the plasmid, the mixture was mixed by pipetting, then the plus reagent was added and the mixture again was mixed gently by pipetting. The mixture was then incubated for 5 minutes RT. The lipofectamine was added last, the mixture was mixed gently by pipetting, and then incubated for further 30 minutes at RT. 100ul of the mixture/well was then added to the plated HEK293T in a drop wise manner and the plate was rocked gently manually and put back again in 37 °C CO2 humidified incubator.

STAT4 Plasmid (0.8 µg)	volume making up 0.8 µg	x #well
Plus	0.8 ul	x #well
Lipo	(2.5*0.8)=2 ul	x #well
DMEM/L-glutamine	100ul	x #well

The cells were inspected for media consumption and old media was replaced at 16-18 hours post transfection using warm full media of DMEM and were then put back in the incubator for a total of ~40 hours.

2.3.2.17 Western blot

Transfected HEK293T (as described above) was left to express the protein before stimulation with IFN-α (20IU/ml or 3000IU/ml) for 1 hr, 5 hours. One well was left without any stimulation for comparison purpose. The activation was terminated at given time by adding cold 1X PBS followed by pelleting the cells at 450g for 5 minutes. The samples were lysed by adding ~60 ul working RIPA buffer / well of 24 well plates incubated for 5 minutes on ice, frozen at -80°C, clarified on the day of the western blot by centrifugation at 8000g for 10minutes.

PBMCs of normal controls and the mutation carrier were plated in ~10million/ml in 24 wells plate and left to rest for 2 hours. This was followed by stimulation with PMA for 1 hour and another well of the same sample was left unstimulated. The stimulation was again terminated at the given time by adding cold PBS and lysing the sample in ~50-60 ul of working cytobuster buffer for ~10 minutes on ice and the supernatant was obtained after centrifugation and frozen at -20°C for future use for western blot.

Protein denaturation of the PBMCs and transfected HEK293T was achieved by adding equal volume of working SDS buffer to the sample, mixing, incubating at 100°C for ~5-7 minutes after which the samples were left to cool down on ice for 5 minutes, finally they were ready to be loaded on SDS- acrylamide gels.

The separation was achieved by running the sample for ~1 hour 100V in a tank filled with working running buffer. The proteins were then transferred to a PVDF membrane (which was briefly activated by soaking it for 1minutes in methanol, 2 minutes in water, and 5 minutes in transfer buffer) and the transfer was done at 100 V for~ 2 hours in chamber filled with chilled transfer buffer. The membrane was then blocked in 3% BSA /TBST 1 hour at RT. The membrane was then stained for the primary antibody in a sealed plastic bag with a total volume of ~3 ml of the specified dilution, overnight at 4°C with very gentle shaking. The next day, the membrane was washed three times of ~20 minutes each on a shaker with TBST. The secondary antibody was then added to the membrane in a sealed plastic bag with a total volume

of ~3 ml of the specified dilution, incubated for 1 hour shaking at RT. The membrane washed again, three times with TBST each 20 minutes on a shaker at RT. The protein detection was done using ECL Western Blotting Detection Reagents, and the strength of the signal was measured by assessing the densitometry relative to the loading (by comparing either GAPDH or TATA box) and the results were expressed in arbitrary units (AU).

2.3.2.18 Flow cytometry

2.3.2.18.1 Surface staining

Cells were resuspended in FACS wash at 1 million /ml and plated in 1-2x10^5cells (200 ul) in 96 well, centrifuged (350g x5 minutes, breaks on) and decanted on ice in the sink to prevent splash. During this time the required antibodies cocktail was prepared by diluting the antibodies in FACS wash according to the optimized dilution for each antibody. At the end of the centrifugation, the plate was then subjected to a quick vortex to dislodge the cells and then ~25 ul of the required pre-diluted surface antibodies was added to each well and incubated for 20 min on ice, washed with FACS wash by adding 150ul/ well, mixing by pipetting, centrifugation (350g x5 minutes, breaks on), decant, vortex and then the wash step was repeated one more time. Single colour compensation tubes were prepared simultaneously. Finally the plate was vortexed, and cells in each well were resuspended with 100 ul /well and transferred into FACS tubes and acquired using FACSCalibure and FACSCantoII. The data were then analyzed with FlowJo, version (8.7) (Treestar).

2.3.2.18.2 Cell sorting

Freshly thawed cells in CM, washed twice in CM and once in sterile plain PBS. The desired cocktail of the antibodies were diluted in PBS supplemented with 0.1 % FCS and 0.5ml of the staining cocktail were added. This was done for cell number of ~50 million and if more cells were needed then the concentration of the antibodies and

volume for staining were adjusted. Single colour compensation tubes were prepared simultaneously. The staining was carried out by incubating the sample for 20 minutes on ice, washed once with plain PBS and finally resuspended in PBS/0.1 % FCS prior to sorting on a FACS ARIA I and/or II cell sorter (BD) under the supervision of MCRF staff.

2.3.2.18.3 Intracellular cytokines detection

Perm wash (cytokine), working Perm wash x1 was prepared on the day
Intracellular cytokines were detected by stimulating 1-2 × 10^6/ ml cells with leukocyte activation cocktail for ~ 4 hours in 24 wells plate. Unstimulated control from the same sample was setup at the same time to control for any background. After 4 hours the cells were harvested, mixed well and dispensed as1- 2x10^5cells (100-200 ul /well) into 96 well plates. Cells were first washed twice with FACS wash, (350g 5 minutes, breaks on), supernatant decanted, plate was vortexed to dislodge the pellet and new FACS wash was added and the process was done one more time. The cells were stained with the desired antibodies using ~25ul of the pre-diluted antibody cocktail / well and left on ice for ~20 minutes. This was followed by washing twice with FACS wash, followed by vortexing before fixing and permabilizing the sample. This was achieved by adding 100ul Cytofix/ Cytoperm, mixing by pipetting, and then the plate was incubated for 30 minutes on ice. The cells were then washed twice with freshly prepared 1x Perm wash, followed by vortex to dislodged the cells before adding the pre-diluted antibodies of the cytokines (dilution was done using Perm wash). The cells were last re-suspended using 100ul/well FACS wash and transferred into FACS tube for acquisition.

2.3.2.18.4 Enumerating Treg, Foxp3 positive/BCL-6

Working Fix buffer (FoxP3 fix) and Working perm solution (FoxP3 perm). Fresh buffers were made on the day and left to reach room temperature.

The cells were surface stained for CD4, CD25 and CD127 as above (surface staining). The cells were fixed in 100 ul of the working buffer A 10 minutes RT away from light , washed once with FACS wash, permed using ~50ul of the working perm buffer ~30 minutes on ice away from light. This was followed by washing the cell twice with FACS wash, staining the cells with pre-diluted Foxp3, 20 minutes on ice, another wash with FACS wash. Finally cells were suspended in fresh FACS wash and transferred into FACS tube for acquisition.

Detecting BCL6 signal was done using the fix and perm conditions for the Foxp3 as outlined above.

2.3.2.18.5 Staining for signalling and transcription factors (STAT1, STAT3, STAT4, T-bet and GATA-3)

Cells were thawed, washed and maintained in sterile CM at a final concentration of 2 × 10^6/ ml. 1- x10^5 cells were plated in 50 ul/ well in sterile 96 well U bottom plates and rested for 2 hours at 37°C, under an atmosphere containing 5% CO2, either stimulated with 50 ul of the indicated cytokine or nothing (adding 50 ul of pre warmed plain CM) for 20 minutes at 37°C incubator, fixed with 100 ul warm fix for 10-15 minutes at 37°C incubators. The plate was centrifuged at 350g for 5 minutes. The supernatant decanted. This was followed by vortex to dislodge the cell pellet at the bottom of the plate, 100 ul of chilled perm solution was added and mixed by pipetting, and the plate was then incubated on ice for ~30 -40 minutes, washed with FACS wash twice and then stained with 25ul/well of pre-diluted antibodies. The samples were then washed one more time with FACS wash and resuspended in100 ul/well and transferred into FACS tube for acquisition.

2.3.2.18.6 Screening TCR signalling

Whole PBMCs were resuspended as 1million/ml and cultured either with nothing or with anti-CD2/CD3/CD28 expansion beads at bead-to-cell ratio 1:2. The plate was

put back in 37 °C incubator for 24 hours. Then the cells were harvested in 96 well plates, washed with FACS wash twice. The cells were stained for surface activation markers using the above surface staining method.

2.3.2.18.7 Detection of PS6(Ser 235/236) by flow cytometry

PBMCS were activated for 3 days with CD3 beads and IL-2. Cells were then washed with CM containing IL-2, activated T cells were separated using beads (CD4 /CD8 microbeads). T cells were either fixed unstimulated or fixed after stimulation with CD3 or CD3 and rapamycin for 10 and 20 minutes.

2.3.2.18.8 T-bet Up- regulation

PBMCs were resuspended in 1million/ml and cultured with anti-CD2/CD3/CD28 expansion beads at bead-to-cell ratio 1:2 with IL-2, IL-12, and with or without rapamycin for 2 days. Then the cells were harvested in 96 well plates, washed with FACS wash twice. The cells were fixed and permeabilised according to the method for the signal transduction and finally stained for T-bet.

2.3.2.18.9 T cell polarization

Th1 [(CD2/3/28 beads at bead-to-cell ratio 1:2) + IL-12, 20 ng/ml+ IL-2, 80ng/ml].
Th2 [(CD2/3/28 beads at bead-to-cell ratio 1:2) + IL-4, 20 µg /ml+ IL-2, 80ng/ml].
Th0 [(CD2/3/28 beads at bead-to-cell ratio 1:2) + IL-2, 80ng/ml].

Naive CD4+ T cells were obtained via FACS sort following the methods outlined for the cell sort (FACS sorted on CD3+CD4+ CD45RO negative), cultured with anti-CD2/CD3/CD28 (bead-to-cell ratio 1:2), ml and desired combination of the desired cytokine for 4 days after which the surface and or the intracellular staining was done following the methods described above.

2.3.2.18.10 Screening BCR signalling

Whole PBMCs were cultured alone or with human CD40L in 24 well plates for 24 hours, after which the sample was harvested, washed twice using FACS wash and surface stained for the given activation markers.

2.3.2.18.11 Plasma cell induction

PBMCS were cultured with IL-21, Type B CpG or CD40L, with and without IFN-γ or anti-IFN-γ for 4 days. After which the cells were harvested, washed twice with FACS wash and stained for the desired combination. Intracellular IgG subclass staining was performed after fixing and perming following the method for cytokine detection.

2.3.2.18.12 Cytotoxicity assay

Cytotoxicity was determined using the PanToxiLux kit according to manufacturer's instructions. Indicated cell populations were incubated with targets cells (labelled TFL4) at various ratios and lethal hit determined by flow cytometry.

2.3.2.18.13 T cell proliferation assays

Indicated cell populations were labelled with Cell Trace Violet (CTV) according to manufacturers. For T cell proliferation, cells were stimulated with T cell activation beads (CD2/3/28) +/- cytokines (as indicated) for 5 days and analysed by flow cytometry.

2.3.2.18.14 Cytokine induction assays

Naive CD4+ T cells (FACS sorted on CD3+CD4+ CD45RO negative) or FACS sorted tonsil CD4T cells according to PD-1 and CD57 were cultured were cultured with anti-

CD2/CD3/CD28 (at a ratio of 2:1) with or with out either recombinant human IL-6 (100ng/ml, Miltenyi Biotec), recombinant Human IL-21(100ng/ml, Miltenyi Biotec), for 2 days. Cells were then analysed by flow cytometry for induction of IL-4 and IL-10. The same experiment was done but the cells were stimulated for 5 days for the CTV experiment. Cells were then analysed by flow cytometry for induction of IL21 and IFN-γ and proliferation.

2.3.2.18.15 Flow cytometry acquisition and analysis

Data were acquired on FACSCaliber or FACSCanto II and data were then analysed with FlowJo, version (8.7) (Treestar).

2.3.2.18.16 Lymph node histology

Frozen sections of human tonsil (6mm) frozen were incubated with indicated fluorescent antibodies for 30 mins then washed and analysed by confocal microscopy.

2.3.2.18.17 RNA-Seq

mRNA libraries for 9 samples were prepared at the Ramaciotti Centre for Genomics UNSW following the standard Illumina protocols. Sequencing was performed on the Illumina NextSeq 500 platform to produce 75 bp reads. There were 3 biological replicates (Tonsil 1, Tonsil 2 and Tonsil 3) for each of the three conditions: (1) PD-1^{hi}CD57+, (2) PD-1^{hi}CD57-, (3) PD-1^{lo}CD57-. The generated reads were mapped to the Ensembl *Homo sapiens* genome (GRCh38) using Tophat2 (v 2.0.12) (D. Kim et al., 2013) calling Bowtie2 (v 2.2.3) (Li and Durbin, 2010). HTSeq-count (Python package HTSeq, python v 2.7.3) (Anders et al., 2015) was used to generate counts of reads uniquely mapped to annotated genes using the GRCm38 annotation gtf file. We performed differential expression analysis using edgeR (v 3.12.0) (Robinson et al., 2010). An MDS plot indicated that samples from Tonsil 2 were outliers. Accordingly, we performed differential expression analysis without Tonsil 2 samples. Low count

transcripts were excluded and only those genes with at least 1 count per million (cpm) in at least 3 samples were used for analysis. Differentially expressed genes were defined as those genes with a Benjamini-Hochberg corrected p value less than 0.1. We then mapped the results back onto the full data set using unsupervised clustering to produce heatmaps comparing expression levels of the genes of interest over all 9 samples.

CHAPTER 3 : HUMAN $STAT4^{P450S}$ IS A NOVEL GAIN OF FUNCTION ALLELE

3.1 Introduction

The overarching aim of the project was to combine clinical, biochemical and detailed cellular data with whole exome sequencing to identify disease pathways in patients with unexplained immune deficiency and inflammatory disease.

3.2 Detailed clinical, biochemical and cellular phenotyping to assist interpretation of whole exome sequencing (WES)

We identified a currently 41 year-old male proband (II.3) who had been diagnosed with bronchiectasis at age of 4 years.

Case history

The proband presented at age 15 months with upper lobe pneumonia. Before this, he had experienced poor feeding, recurrent purulent nasal discharge and failure to thrive. Investigations at this time revealed opacification of the maxillary antra bilaterally, and right upper lobe consolidation. Sputum culture yielded *H. influenzae*. Immunoglobulin studies revealed an elevated IgA (2.87 g/L, normal range: 0.6-0.8), IgG at the upper limit of normal (10.0g/L, normal range: 8.0-10.0) and normal IgM (1.07g/L, normal range: 0.6-0.85). He had a normal sweat chloride concentration. He had mild hypochromic microcytic anaemia and iron deficiency and one episode of hematemesis.

He had several similar presentations over the next 18 months and was diagnosed with bronchiectasis at age 4ys. He had chronic nasal discharge. He developed a chronic macular eruption over his arms and legs.

He was treated with regular chest physiotherapy and postural drainage. He required frequent courses of antibiotics for *H. influenzae* chest infections. At the age of 2 his immunoglobulin studies were repeated and revealed IgA 1.2g/L (0.27-1.92), IgM 1.1g/L (0.41-1.68) and IgG 17g/L (4.0-16.2). Ultra-structural studies of nasal cilia were normal.

He suffered recurrent infections throughout adolescence. He was small for his age. He also suffered from symptoms thought to be consistent with reflux or achalasia in adolescence. At the age of 16 he experienced an oesophageal obstruction from a piece of meat that was retrieved at gastroscopy. This study also revealed inflamed and friable oesophageal mucosa. A radionuclide swallowing study showed a hold up of the tracer in the upper third of the oesophagus. Maximum tracer clearance was 76% and was delayed (>18 sec) consistent with mild-moderate oesophageal dysmotility disorder.

At age 18, investigations revealed IgG 15.5g/L and IgA 7.8g/L. IgM and IgE were normal. He was deficient for IgG2 (0.35, normal range: 1.95-4.5) and IgG4 (0.06, normal range: 0.15-0.3) while IgG1 (10, normal range: 3.9-9.0) and IgG3 (4.69 normal range: 0.4-0.9) were elevated. His specific antibody titres to tetanus toxoid and diphtheria were low but detectable after vaccination.

He was commenced on intravenous immunoglobulin replacement and this resulted in a reduced frequency of chest infections. He experienced ongoing symptoms of gastro-oesophageal reflux and non-specific dermatitis. His only other inter current infection was *Trichophyton mentagrophytes* onychomycosis.

We initiated our investigations by a detailed assessment of different T and B cells subsets. T cell analysis revealed a marked CD8+ T cell expansion, which placed him outside of the distribution of other patients with antibody deficiency (Figure 3.1 A, upper row and Figure3.1B). More detailed analysis showed that the CD8+ T cell expansion was mainly due to an increase in the CD45RA+ effector subset (Temra,

CD45RA+ CCR7-) (Figure 3.1 C-D).We also found him to have a partial memory B cell deficiency (Figure 3.1 A, lower row).

Next we tested for any abnormalities in proximal T and B cell signalling through examination of up regulation of early activation markers after short term stimulation. CD25 (Waldmann 1986; Robb, Munck, and Smith 1981) and CD69 were used as early activation markers in T cells(Testi, Phillips, and Lanier 1989) and CD69 and CD83 were used as readout for early B cell activation (Kretschmer et al. 2007).The difference between 24 hours post stimulation compared to the baseline of the activation markers after *in vitro* stimulation of PBMCs with CD2/3/28 labelled beads for T cell activation or with anti-Ig and CD40L for B cell activation, was examined by flow cytometry. We found no evidence for a defect in proximal T or B cell activation (Figure 3.1 E-H) in the proband compared to the controls.

Pathological actions of STAT4, MTOR and STAT3 in human T cell differentiation

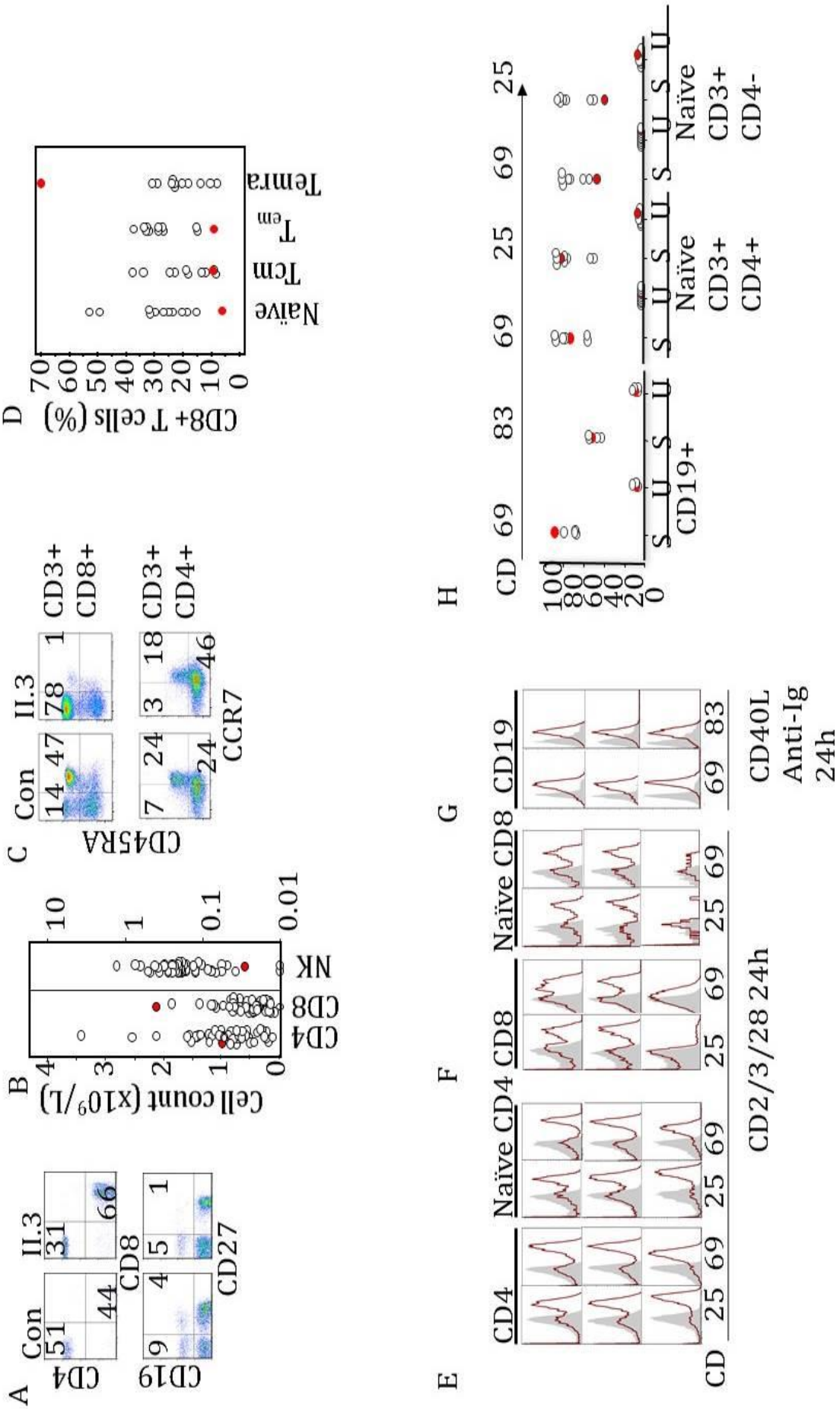

Figure 3-1 Detailed T and B cell phenotype

A. Representative flow cytometry analysis of circulating CD4+, CD8+T cells , naïve B(CD19+CD27-) and memory B cells (CD19+CD27+) in controls and proband (II.3). **B.** Summary of absolute counts of circulating CD4+, CD8+ T cells and NK cell (CD16/56) obtained by flow cytometry in II.3(Red) and patients with antibody deficiency(n=50). **C.** Representative flow cytometry analysis of different subsets in CD8+ T cells; Temra (CD45RA +CCR7-), Naïve (CD45RA+CCR7+), Tcm (CD45RA-CCR7+), Tem (CD45RA-CCR7-) in control (con) and II.3. **D**. Summary of circulating CD8+ T subsets as a percentage of CD3+ T cells. II.3 (red filled circle) and controls, n =11 (unfilled circles). **E-F** Overlay histograms of T cell activation markers (CD25 and CD69).PBMCs were cultured with CD2/3/28 beads (1: 2 ratio) for 24 hours. T cells were analysed by flow cytometry for CD25 and CD69 up-regulation among total CD4+(CD3+CD4+)T cells and naive gated CD4+ T cells (CD45RA+CCR7+) and among total CD8+ and naïve gated CD8+ (CD3+CD8+) T cells (CD45RA+CCR7+) at baseline (grey filled histogram) and 24hrs post stimulation (red unfilled histogram). **G**. Overlay histogram of B cell activation markers (CD69 and CD83).PBMCs were cultured with anti-IgM and CD40L for 24hrs, then B cells were analysed for activation by flow-cytometry according to induction of CD69 and CD83 on CD19 gated B cells at baseline (grey filled histogram) and 24hrs post stimulation (red unfilled histogram). **H**. Summary of percentage of up-regulation of different activation markers in T and B cells in II.3 (red filled circle) versus controls (unfilled circles, n=7).

We showed in the previous section that the proband (II.3) exhibits a deviation toward effector differentiation mainly in CD8+ T cells as assessed by the surface phenotype (Figure 3.1 C-D). Next, we examined II.3 T cells for effector differentiation according to cytokine expression. The CD8+ T cell compartment exhibited marked deviation to IFN-γ production (Figure 3.2 A-B). In particular, we observed substantial IFN-γ production by the Temra subset in the CD8+ T cell compartment (Figure 3.2 C).Despite the normal distribution of memory and naïve cells in the CD4+ T cell

compartment (Figure 3.1 C), II.3 also exhibited deviation to IFN-γ production with the CD4+ T cell compartment (ie Th1 deviation) evident by cytokine (IFN-γ) expression as well as level of chemokine receptor (CXCR3+CCR6-) (Figure 3.2 D-F). By contrast, *ex vivo* enumeration of Th2 (IL-4, IL10) (Figure 3.2 E), Th17 (IL-17) (Figure 3.2 F) and T regulatory (Treg) ($CD25^{high}$ $CD127^{lo}$, Foxp3+) (Figure 3.2 G) revealed similar results r to those from normal controls.

The observed Th1 deviation expressed by CD4+ and CD8+ T cells in II.3 might indicate an intrinsic propensity of T cells to adopt Th1 phenotype. To investigate this possibility further, we examined the ability of naïve CD4+ T cells (from II.3) to adopt this extreme Th1 differentiation under Th1 enriching environment. Naïve CD4+ T cells were sorted using fluorescence activated cell sorting (FACS) sorting from the proband (II.3) and a control. FACS sorted naïve CD4+ T cells were then cultured for four days under Th1 (CD2/3/28+IL-12) or Th2 (CD2/3/28+IL-4) polarizing conditions. Naïve CD4+ T cells from the proband (II.3) cultured under Th2 did not express excess IFN-γ relative to control cells from a healthy donor(Figure 3.2 H, lower row). By contrast, when naïve CD4+ T cells from II.3 cultured under Th1 conditions, they exhibited marked Th1 deviation (Figure 3.2 H, upper row) compared to the normal control cultured under the same Th1 conditions.

Pathological actions of STAT4, MTOR and STAT3 in human T cell differentiation

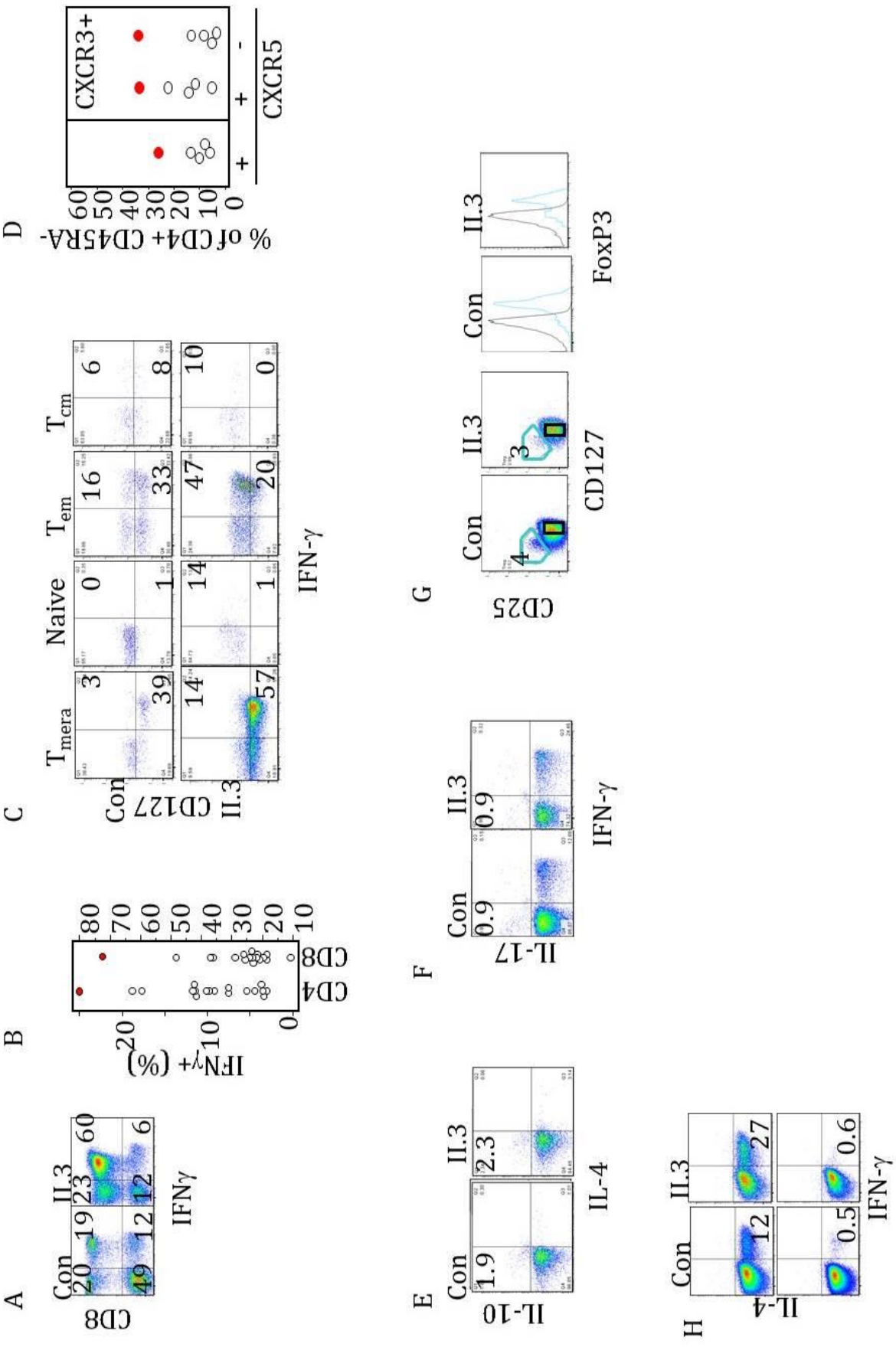

Figure 3-2 Expanded IFN- γ production by CD4+ and CD8+ T cells

A. Representative flow cytometry analysis of intracellular IFN-γ expression by CD8+ T and CD4 +T cells in II.3 and a normal control after 4 hours stimulation with PMA and ionomycin and brefeldin-A. **B**. Summary of IFN-γ producing cells as a percentage of total CD4+ and CD8 + T cells. II.3 (*red filled circle*) and controls, n=17 (*unfilled circles*). **C**. Flow cytometry analysis of intracellular IFN-γ expression by sorted CD8+ T cell subsets and stimulated for 4 four days with + CD2/3/28 beads (1: 2 ratio) + IL-12(0.2ng/ml). **D.** Representative flow cytometry analysis of Th1 by chemokines expression (CXCR3+ CCR6-) in CD4+ T cells in II.3 and normal controls (n=4). **E**. Representative flow cytometry analysis of intracellular IL10 and IL-4 expression by CD4+ T cells in II.3 and a normal control after 4 hours stimulation with PMA and ionomycin and brefeldin-A. **F**. Representative flow cytometric analysis of intracellular IL-17 and IFN-γ expression by CD4+ T in II. 3 and a normal control after 4 hours stimulation with PMA and ionomycin and brefeldin-A. **G**. Representative flow cytometric analysis of T regulatory cells using surface phenotype of ($CD127^{low}$, $CD25^{high}$) then gating on intracellular Foxp3+(histogram; black unfilled gated on effector CD127 positive and blue unfilled gated on $CD127^{low}$, $CD25^{high}$ in II.3 and a normal control. **H**. Flow cytometry analysis of intracellular IFN-γ expression by induced Th1 in the upper row [(CD2/3/28 beads at bead-to-cell ratio 1:2) + IL-12, 20 ng/ml+ IL-2, 80ng/ml] and Th2 in the lower row[(CD2/3/28 beads at bead-to-cell ratio 1:2) + IL-4, 20 µg /ml+ IL-2, 80ng/ml] cells from naïve precursors FACS sorted on CD3+CD4+CDR5RO- after 4 days cultured under polarising conditions in II.3 and a normal control.

This is an exaggeration of the normal response, since Th1 deviation was only observed when naïve cells were activated under Th1 conditions, with IL-12 (Figure. 3.2H, upper row), but not when stimulated with IL-4 (Figure. 3.2H, lower row).

In summary, the most remarkable cellular phenotype identified in the proband (II.3) is deviation to IFN-γ production by both CD4+ and CD8+ T cells. This deviation appears to be cytokine-dependent, and not spontaneous.

3.3 Analysis of whole exome sequence

The proband (II.3) underwent a whole exome sequencing (WES) as part of our primary antibody deficiency discovery program (ANZADA project). We obtained >30X coverage for >96% of the coding genome. Our data analysis pipeline incorporates information obtained from dbSNP, OMIM, Gene ontology, and ExAC databases and the ImmGen expression database. This provided an initial filter, based on prevalence of the identified variants, known associated phenotypes, and relevance to immunity. In addition, we obtained PolyPhen-2 and SIFT scores for each nonsynonymous variant.

Whole exome sequencing of the proband revealed 8511 non-synonymous single nucleotide variants, of which 116 were novel at the time of writing (Figure 3.3A). We typically assign scores to each variant that takes into account frequency, expression, in silico estimates of likelihood of damage and known disease associations, and by this tentative approach, a novel heterozygous variant in *STAT4* yielded the highest score (Figure. 3.3A-B).

This single nucleotide *STAT4* variant encodes a proline to serine substitution at position 450 (P450S; chr2:191904011G>A; g.112312C>T; c.1348 C>T), and has not been reported in TGP, dbSNP, ExAC, ClinVar or HGMD and has not been detected in >300 in-house exome sequences from unrelated individuals. Proline-450 is a highly conserved residue (Figure. 3.3C) located in the DNA binding domain and the substitution with serine is predicted to be damaging by polyphen2, SIFT and mutation taster (Figure. 3.3A-B).

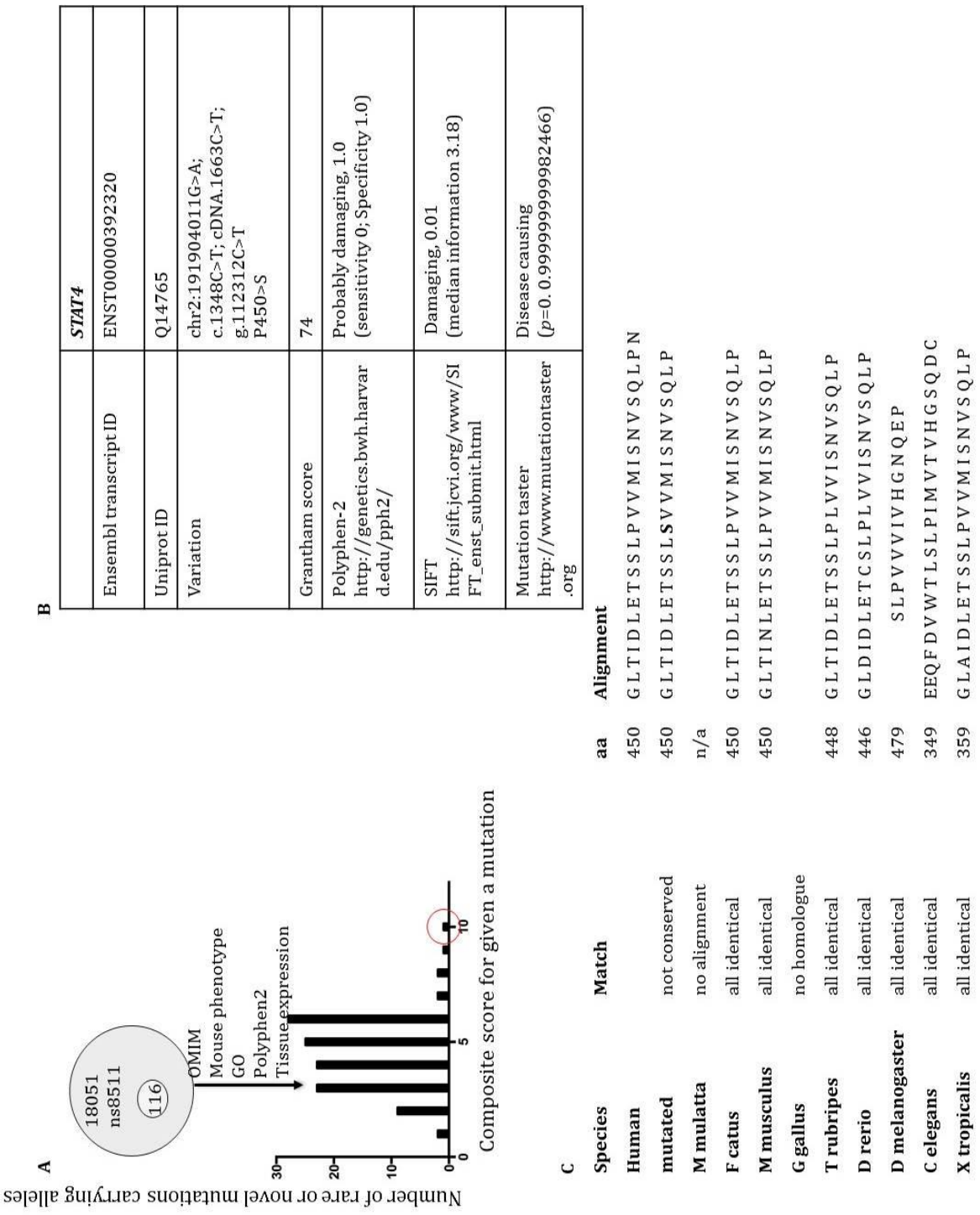

Figure 3-3 *STAT4* ^{P450S} score

A . Summary of whole exome sequence of proband and the filter score obtained for novel missense mutations (*STAT4* score circled). **B.** Characteristics of *STAT4*$^{P450>S}$

mutation and some of the used scores. **C** Conservation of mutated base and amino acid in *STAT4* across different species (from Mutation taster).

STAT4$^{P450>S}$ was confirmed by Sanger sequencing (Figure. 3.4 A-B). The rest of the available family members were also genotyped for this mutation and we identified one sibling and one of her offspring with the same mutation (Figure. 3.4 C-D).

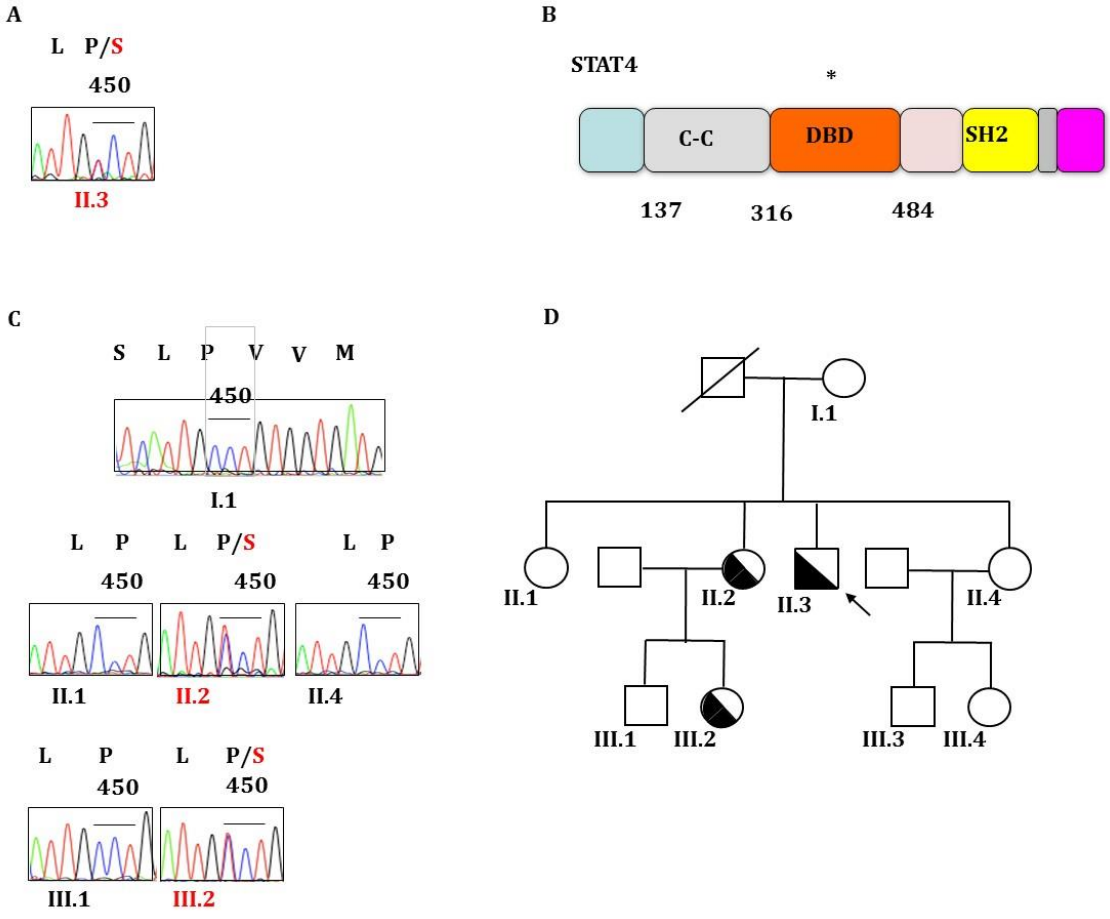

Figure 3-4 Confirmation of STAT4P450>S on available family members

A. Electropherogram of *STAT4* $^{P450>S}$ in the proband (II.3). **B.** Cartoon of different domains of STAT4. The location of P450 is shown (*) in the DNA binding domain (DBD). C-C, coil- coil domain, SH-2 domain **C.** Electropherogram of *STAT4* in the available family members and **D.** family pedigree of the available family members

indicating the one with *STAT4* $^{P450>S}$**;** wild type family members (unfilled), un genotyped dead father (unfilled with crossing line), members carrying heterozygous *STAT4* $^{P450>S}$ (half- filled circle) and the proband carrying heterozygous *STAT4* $^{P450>S}$ (half-filled square and an arrow).

As noted in the introduction (Chapter 2.2- 2.4), the DNA binding domain of STAT4 is crucial for recognition and binding to the DNA responsive element in the nucleus, including IFN-γ and IRF1 (Horvath, Wen, and Darnell 1995; Xu, Sun, and Hoey 1996). The DNA binding domain of STAT4 is similar to that of STAT1 (Xu, Sun, and Hoey 1996). Mutation within this domain does not alter the ability to activate and phosphorylate the STAT1 molecules (Horvath, Wen, and Darnell 1995; Meyer et al. 2003). Moreover, binding of pSTAT1 dimers to the DNA domain protects it from dephosphorylation (Takezaki et al. 2012; Meyer et al. 2003). Based on this, we predicted that *STAT4* $^{P450>S}$ would confer a gain-of- function.

3.4 IL-12 STAT4 activation is necessary for Th1 deviation

Phosphorylation after activation induces a conformational change in the STAT4 homodimer (Meyer et al. 2003), which enhances recruitment to its nuclear binding elements, including regulatory elements of *IRF-1 and IFN-G* genes (Xu, Sun, and Hoey 1996). Binding of the STAT4 dimer to its nuclear element, results in relative protection from de-phosphorylation (Meyer et al. 2003). We noted in the previous section that CD4+ T cells from II.3 exhibited cytokine (IL-12) dependent Th1 deviation (Figure 3.2H). IL-12 is crucial for naïve CD4+ T cells to adopt a Th1 phenotype through recruitment and activation of STAT4 (Yamamoto et al. 1994; Zhong, Wen, and Darnell 1994; Kaplan, Sun, et al. 1996) and Th1 induction by IL-12 is abrogated by STAT4 deficiency in mice (Kaplan, Sun, et al. 1996; Carter and Murphy 1999) (Chapter 2.3 and 2.8).

IFN-γ is only induced at low levels in naïve CD4+ and CD8+ T cells after stimulation by TCR (Kaplan, Wurster, and Grusby 1998) (Figure 3.5 A). Optimal induction in

both naïve CD4+ and CD8+T cells of IFN- γ requires addition of IL-12 (Figure 3.5 B). Moreover, stimulation of resting PBMCs cells with IL-12 does not activate STAT4 (Figure 3.5 C) due to the lack of expression of inducible IL12Rβ2 subunit of the IL-12 receptor on resting T cells (Igarashi et al. 1998; Ahlers et al. 2001) as well as low level of STAT4 expression by resting lymphocytes. Up-regulation of the expression of both IL12Rβ2 and STAT4 is induced by T cell activation with either TCR and co stimulation (CD2/3/28) or mitogen (PHA). After this, cells respond to IL-12 with phosphorylation of STAT4 (pSTAT4). Thus, activation of PBMCs from normal controls with either PHA or CD2/3/28 followed by stimulation with IL-12 or IFN-α, resulted in activation and phosphorylation of STAT4(pSTAT4) (Figure 3.5 D-E). Of note, stimulation of IL-12 can maintain a longer phosphorylation of STAT4 compared to IFN- α (Figure 3.5D-E). Activation of STAT4 by IL-12 also resulted in up-regulation of T-bet (Figure 3.5 F) which is crucial to maintain Th1 differentiation (IFN- γ) (Figure 3.5 B)(Cho et al. 1996; Yamamoto et al. 1994; White et al. 2001; Jacobson et al. 1995; Kaplan, Sun, et al. 1996; Testi, Phillips, and Lanier 1989)

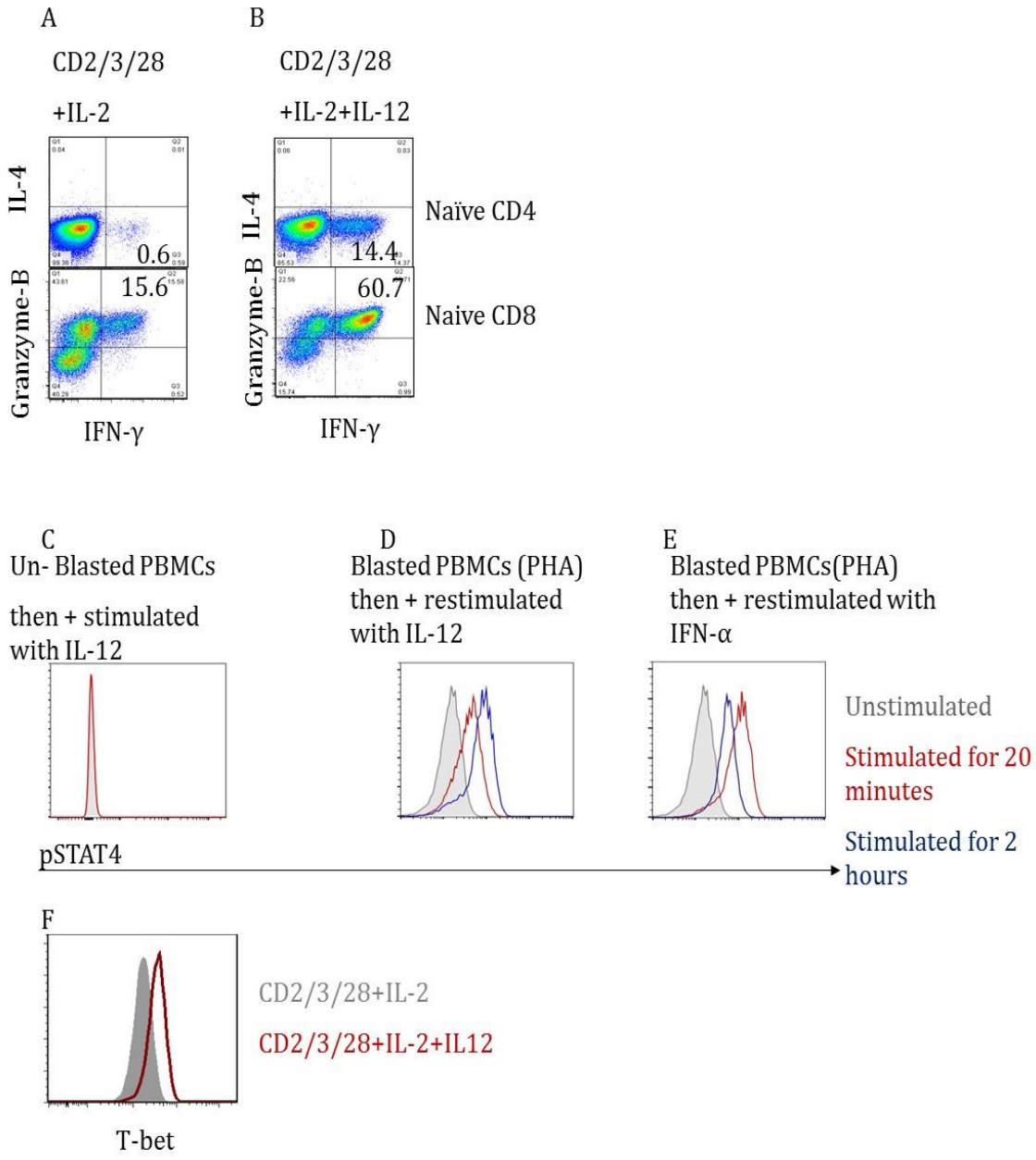

Figure 3-5 IL-12 STAT4 dependent Th1 formation

A-B. FACS sorted naïve CD4+ T cells (CD3+CD4+CD45RO-) and CD8+ T cells (CD3+CD4+CD45RA+CCR7+) from a healthy donor were cultured for 4 days with CD2/CD3/28 + IL 2 (80ng/ml) as in **A** or CD2/CD3/CD28 +IL-2 (80ng/ml) + IL- 12 (20ng/ml) as in **B**. Cells were then analysed by flow cytometry analysis for intracellular IFN-γ and IL-4 expression (CD4+ T cells) and granzyme-B and IFN- γ

(CD8+ T cells). **C.** Overlay histogram of pSTAT4 expression in *ex vivo* PBMCs detected by flow cytometry in unstimulated (grey filled) versus stimulated for 20 minutes with IL-12 (20ng/ml, unfilled red histogram). **D.** Overlay histogram of pSTAT4 expression detected by flow cytometry in blasted PBMCs with PHA (25 µg /ml) for 48 hours then either left unstimulated (grey filled) or re stimulated either for 20 minutes(unfilled red) or for 2 hours (unfilled blue) with IL-12(20ng/ml). **E.** Overlay histogram of pSTAT4 expression detected by flow cytometry in blasted PBMCs with PHA (25 µg /ml) for 48 hours then either left unstimulated (grey filled) or re stimulated for either for 20 minutes(unfilled red) or for 2 hours (unfilled blue) with IFN-α(1000U/ml). PBMCs in both conditions were obtained from the same healthy donor. **F.** Overlay histogram of T-bet expression determined by flow cytometry. Naïve CD4+ T cells were stimulated for 4 days with either CD2/3/28 or IL-2 (80ng/ml, grey filled) or with CD2/3/28+IL-2 (80ng/ml) +IL-12 (20ng/ml, red unfilled). Naïve cells in both conditions were obtained from the same healthy donor.

3.5 Novel STAT4 P450S is associated with cellular phenotype of deviated Th1 and IFN- γ expression

We identified the mutation encoding $STAT4^{P450S}$ in one out of three siblings of the proband (II.2) (Figure. 3.4C-D), and she remains clinically healthy with no evidence of bronchiectasis, oesophagitis or dermatitis. Nonetheless, we proceeded to characterize her lymphocyte phenotype. This revealed a normal distribution of CD4+ and CD8+ T cells and their subsets (Figure 3.6 A-B), and normal numbers of NK cells. She also exhibited normal B cells subsets (Figure 3.6C), normal serum immunoglobulin, and normal IgG subclass distribution. *In vitro* studies revealed normal plasma cell induction (Figure 3.11). We then examined T cells from all available siblings for deviation in effector cytokine production and differentiation.

Ex vivo analysis of the circulating effector T cell subsets in the $STAT4^{P450S}$ sibling revealed no abnormality of Th2, and Th17 or Treg differentiation. However, there was some heightened Th1 deviation as assessed by proportion of IFN-γ + CD4+ T

cells (Figure 3.6D). Next, we examined the propensity of naïve CD4+ T cells to deviate toward Th1 cells after activation *in vitro*. In contrast with ex vivo analysis, naïve CD4+ T cells from the $STAT4^{P450S}$ sibling exhibited very clearly an enhanced propensity to IFN-γ formation after stimulation under Th1 conditions (Figure 3.6E upper row). Similar to II.3, Th1 deviation was cytokine dependent as naïve CD4+T cells cultured with CD2/3/28 beads alone failed to adopt a Th1 phenotype (Figure 3.6E, lower row).

Pathological actions of STAT4, MTOR and STAT3 in human T cell differentiation

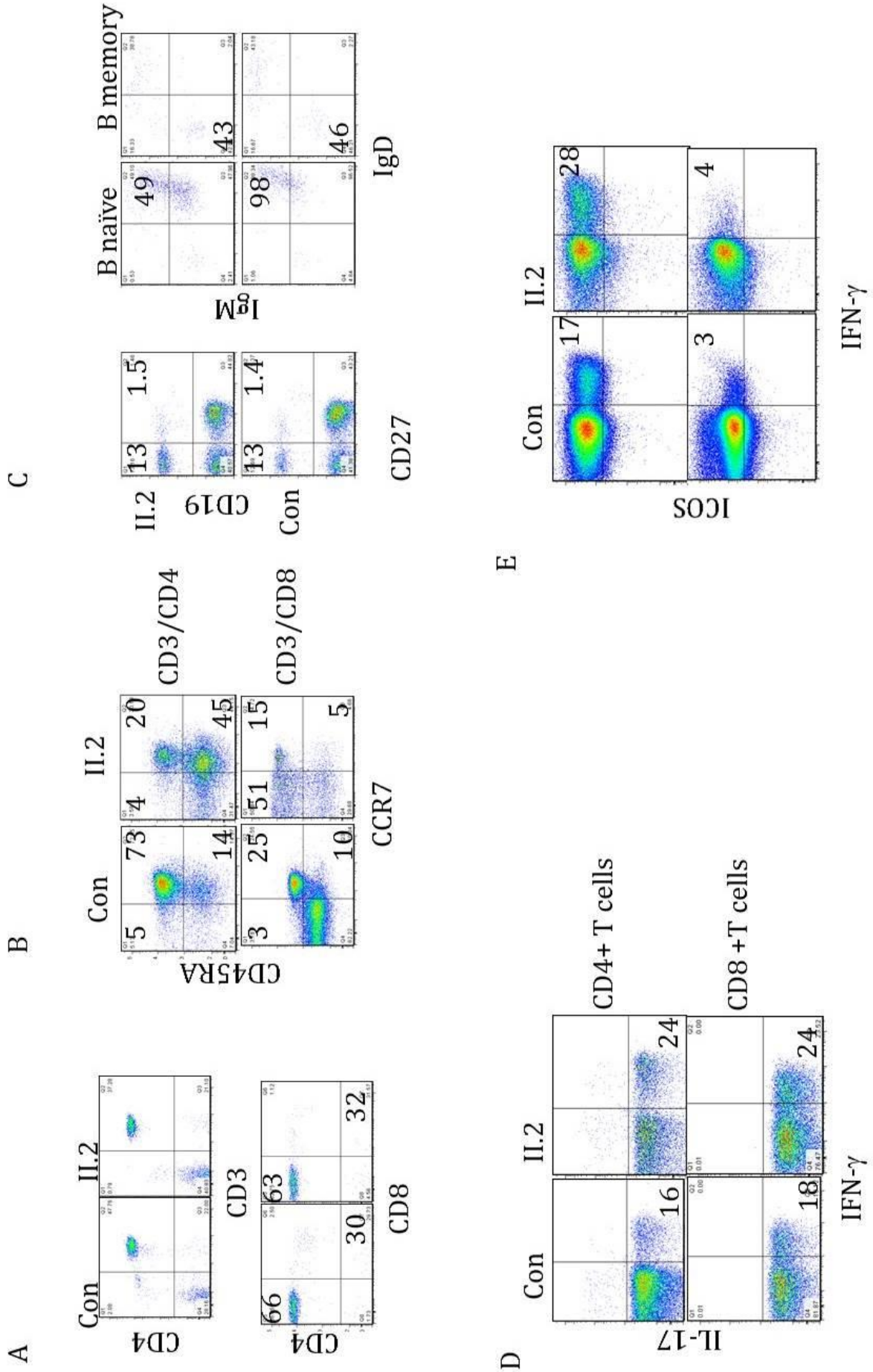

Figure 3-6 Enhanced propensity toward Th1 deviation exhibited by carrier of *STAT4*^{P450S}

A. Flow cytometry analysis of circulating CD3+ T cell subsets in a control and (II.2). **B.** Flow cytometry analysis of circulating CD4+ and CD8 + T cells subsets according to CD45RA and CCR7 expression in a control and (II.2).**C.** Representative flow cytometry analysis of circulating B naïve(CD19+CD27-) and memory ells(CD19+CD27+) in a control and (II.2) and the surface IgM and IgD expression in each of these two subsets. **D.** Representative flow cytometry analysis of intracellular IL-17 and IFN-γ in CD4+T cells in a control and (II.2) after 4 hours stimulation with PMA and ionomycin and brefeldin-A. **E.** Flow cytometry analysis of intracellular IFN-γ expression in polarized CD4+ T cells after 4 days of culturing naïve CD4+T cells (CD3+CD4+CD45RO-) in a control and (II.2) with CD2/3/28 + IL-2 (80ng/ml) +IL-12 (20ng/ml ,upper row) or only with CD2/3/28+IL-2 (80ng/ml, lower row).The experiment has been done at least twice.

3.6 Novel STAT4P450S missense mutation confers gain-of-function

3.6.1 *In-vitro* analysis of PBMCs

Mutations involving DNA binding domain in STAT1 protect it from dephosporylation and result in gain of function of STAT1 (Takezaki et al. 2012; Yamazaki et al. 2014; Frans et al. 2014; Faitelson et al. 2014; Uzel et al. 2013; Liu et al. 2011). We considered that a similar action might be conferred by the novel mutation in *STAT4* discovered and described here. In order to test this postulate, we evaluated STAT4 phosphorylation (Y693) in naïve CD4+ T cells cultured under Th1 conditions. FACS sorted naïve CD4+ T cells from II.2 (*STAT4*^{P450S}*)* and cells from two healthy controls were cultured under Th1 inducing conditions (CD2/3/28 + IL-2+ IL-12) or under neutral conditions (CD2/3/28 +IL-2) for four days. We observed that only in cells bearing the *STAT4*^{P450S} variant, cytokine-dependent phosphorylation of STAT4 was maintained for a longer time (Figure 3.7 A).

3.6.2 *In-vitro* analysis of *STAT4*^{P450S} transfected HEK293T

3.6.2.1 Cloning and expression of STAT4^{P450S}

In order to investigate the function of the *STAT4*^{P450S} allele in more detail and to eliminate any possibility of another mutation in II.3 or II.2 contributing to or modifying the prolonged pSTAT4, we set out to clone both normal and mutant alleles. Moreover, *STAT4* is expressed as two isoforms, α and β. We were also interested in investigating whether the *STAT4*^{P450} mutation has led to alteration in relative expression of mutant or normal allele, or relative expression of either of the two isoforms.

Since the II.3 is heterozygous for *STAT4*^{P450S}, we aimed to clone both alleles from II.3 PBMCs. Detailed method is described earlier (Chapter 2.3.2.3- 2.3.2.13). Briefly, RNA was isolated from PBMCs of (II.3), and reverse transcribed. *STAT4* cDNA was amplified from this template using high fidelity DNA polymerase (Phusion Hot Start II High-Fidelity DNA Polymerase). Amplified *STAT4* was then transformed into MAX Efficiency® DH5α™ Competent Cells. Plasmids from positive colonies were extracted and sequenced. Colonies expressing mutant and wild type (WT) *STAT4* were found with similar frequency, indicating expression of both wild type and mutant alleles in similar proportion in patient's cell. We also identified that both common isoforms (splice variants) of *STAT4* (Hoey et al. 2003) were found with either WT or g.112312C>T genotypes.

Next, we tested the functional consequences of *STAT4*^{P450S} (Chapter 2.3.2.13-2.3.2.16). Briefly, we transfected HEK293T cells with either normal or *STAT4*^{P450S} alleles (HEK293T do not express endogenous *STAT4*). Moreover, HEK293T do not express endogenous IL-12R and therefore, we used IFN-α to stimulate transfected cells to investigate the functional consequences of *STAT4*^{P450S} on inducing prolonged phosphorylation which was evaluated by flow cytometry, western blot, and

immunofluorescence. Furthermore, luciferase was used to assess the transcriptional consequences after stimulation of the mutant allele compared to the wild allele.

3.6.2.2 $STAT4^{P450S}$ is associated with prolonged phosphorylation and nuclear localization

First, we attempted to replicate persistence of pSTAT4 seen on freshly polarised CD4+ Th1 cells using transfected HEK293T. Based on published findings (Hoey et al. 2003), isoform β (shorter) maintains a prolonged phosphorylation state compared to the α isoform. Therefore, we proceeded with our phosphorylation experiments by flow cytometry and western blot using the β isoforms of wild type and mutant alleles. Transfected HEK293T cells were stimulated with IFN-α for 2 days, and then rested in normal media for another 2 days with no further stimulus. Cells were then examined for phosphorylation of STAT4. Cells transfected with the $STAT4^{P450S}$ showed sustained pSTAT4 compared to those transfected with wild *STAT4* (Figure 3.7 B), replicating the findings from induced Th1 cells (Figure 3.7 A).

Analysis of transfectants for abundance of pSTAT4 was performed by western blot. The signal obtained for pSTAT4 was normalized to the MYC (to control for the efficiency of the transfection) and TATA box (to control for the efficiency of protein loading) and then this signal was expressed relative to the unstimulated cells. The analysis revealed sustained phosphorylation after 1 and 5 hours in response to IFN-α stimulation which was most evident in response to low-dose stimulation. The differential response was evident at both time points, with a greater difference at the later time point (Figure 3.7 C-D).

Next, we examined the distribution of STAT4 in transfected cells by direct immunofluorescence. This revealed a higher proportion of nuclear STAT4+ cells in mutant transfectants after overnight stimulation with IFN-α (Figure3.7E) .

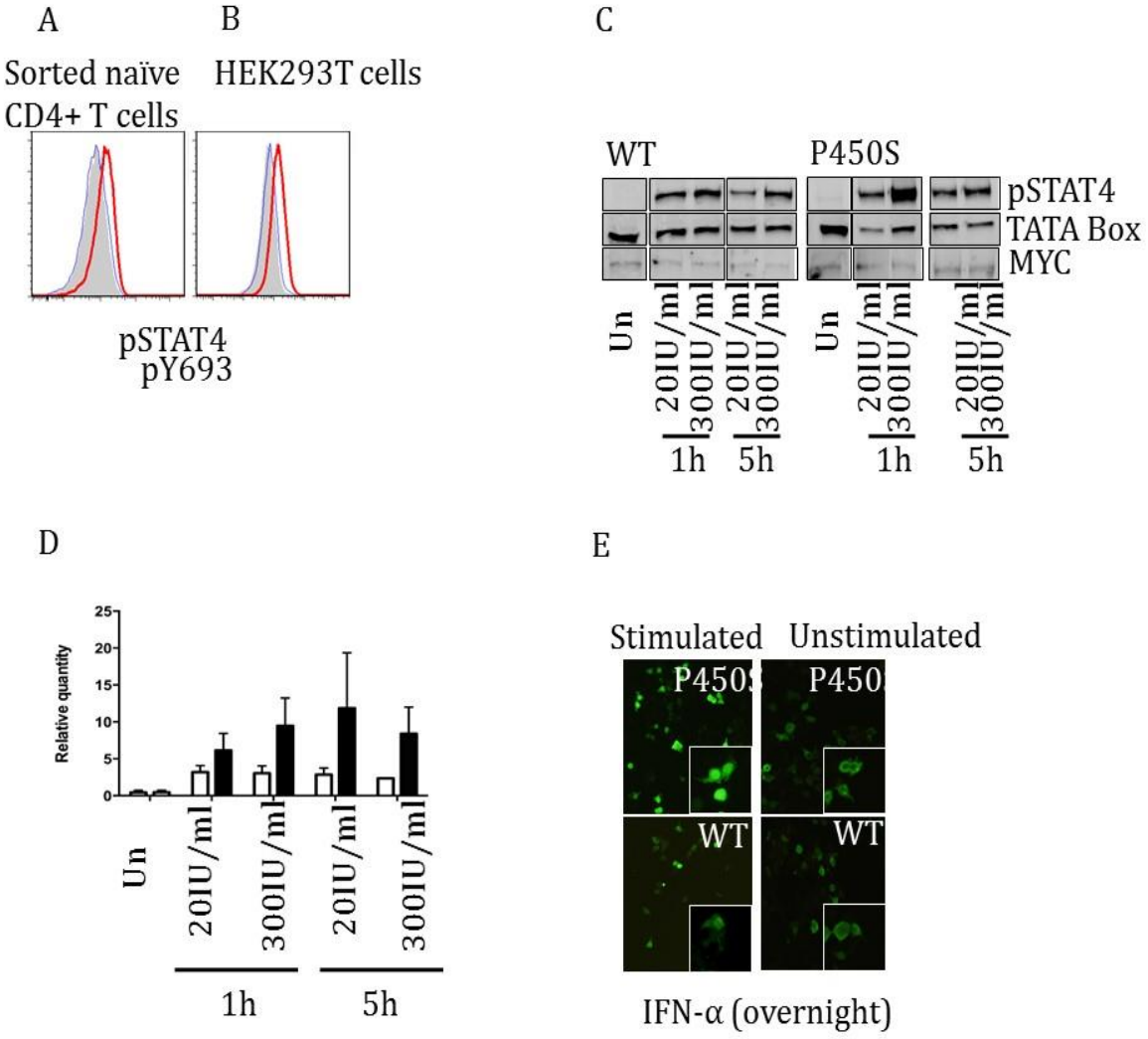

Figure 3-7 STAT4 P450S is associated with prolonged phosphorylation and nuclear localization

A. Overlay histogram of level of pSTAT4 in naïve CD4+ T cells (CD3+CD4+CD45RO-) cultured for 4 days with CD2/3/28+ IL-2 (80ng/ml) +IL-12 (20ng/ml) in II.2 (red unfilled) versus normal control (filled grey) and sibling with wild type *STAT4* genotype (unfilled blue). Cells were washed, fixed, permabilised and then stained for pSTAT4 staining. **B.** Overlay histogram of level of pSTAT4 in un transfected HEK293T (filled grey), transfected HEK293T with wild allele of *STAT4* (blue unfilled, β isoform) or transfected HEK293T with mutant allele of *STAT4* (red unfilled, *STAT4* β isoform). The three constructs were stimulated with IFN-α

(64IU/ml) for 2 days, washed and rested in culture media with no further stimulation for another 2 days. Then, cells were washed, fixed, permabilised and stained for pSTAT4. **C.** Western blots of pSTAT of HEK293T transfected with β isoform of wild (WT) or mutant type (*P450S*). The level of pSTAT4 by western blot was assessed at time 0, 1 and 5 hours of activation. Staining was done for pSTAT4, TAT Box as internal control and MYC as a control for the ratio of transfection. Cells were stimulated with low dose of IFN- α (20 IU /ml) versus high dose of (3000 IU /ml). **D.** Summary of level of pSTAT4 relative quantity after normalisation for the transfection efficiency using MYC and for the level of denaturation by TATA Box in wild type transfected HEK293T (unfilled columns) and mutant HEK293T (black filled columns). **E.** Transfected HEK293T with β isoform were stimulated with IFN- α overnight (64 IU/ml), washed, stained and subjected to examination by fluorescent microscope to assess the degree of nuclear localisation. Each experiment has been done once but under different conditions and using many different controls (minimum of two) to validate the given result.

3.6.2.3 $STAT4^{P450S}$ is associated with prolonged transcription

STAT4 is activated after stimulation with IFN- α or IL-12. Moreover, expression of IRF1 is transcriptionally dependent on activation of STAT4. Mice deficient in either IRF1 (Coccia et al. 1999; Galon et al. 1999) or STAT4 (Kaplan, Sun, et al. 1996) have impaired Th1 development. Therefore, to assess the transcriptional consequence of $STAT4^{P450S}$, we co-transfected *STAT4* luciferase reporter vector (*IRF1*) and pRL Renilla luciferace reporter vector (*pRL-CMV* vector) into HEK293T cells, together with either mutant or wild type and either alpha isoform or β isoform *STAT4* cDNA plasmids. *CMV pRL* was co transfected as a control reporter (renilla signal). At ~36-40 hours after transfection (sufficient time for protein expression), HEK293T cells were stimulated with IFN-α (20IU/ml vs. 3000IU/ml) for 6 or 24h. Luciferase induction was terminated at 6 or 24 hours by washing the cells with phosphate buffered solution (PBS), and luciferase activity was assessed immediately by luminometry (Dual-Glo Luciferase Assay System). The signal obtained from the

experimental reporter (*IRF1*) was then normalised to the renilla (control reporter) and then this signal was expressed relative to the unstimulated cells.

In cells transfected with β isoform of *STAT4*, we observed a statistically significant prolonged and increased IRF1 signal in the presence of the mutant *STAT4* allele compared to the wild type. This was apparent in response to both the low-dose and the high dose stimulation with IFN- α, and it was cytokine-dependent (Figure 3.8.A-B). Moreover, it was significant at 6 hours and remained significant 24 hours later. Interestingly, transfection with α isoform resulted in a high signal in cells transfected with the mutant allele compared with wild type allele, in response to stimulation to either low dose or high dose of IFN- α but only in the 6 hours duration(Figure 3.8.C-D). At 24 hours, this difference was lost (Figure 3.8.C-D). These results are consistent with previous studies which have reported that the β isoform maintains phosphorylation and therefore transcription for a longer time (Hoey et al. 2003).

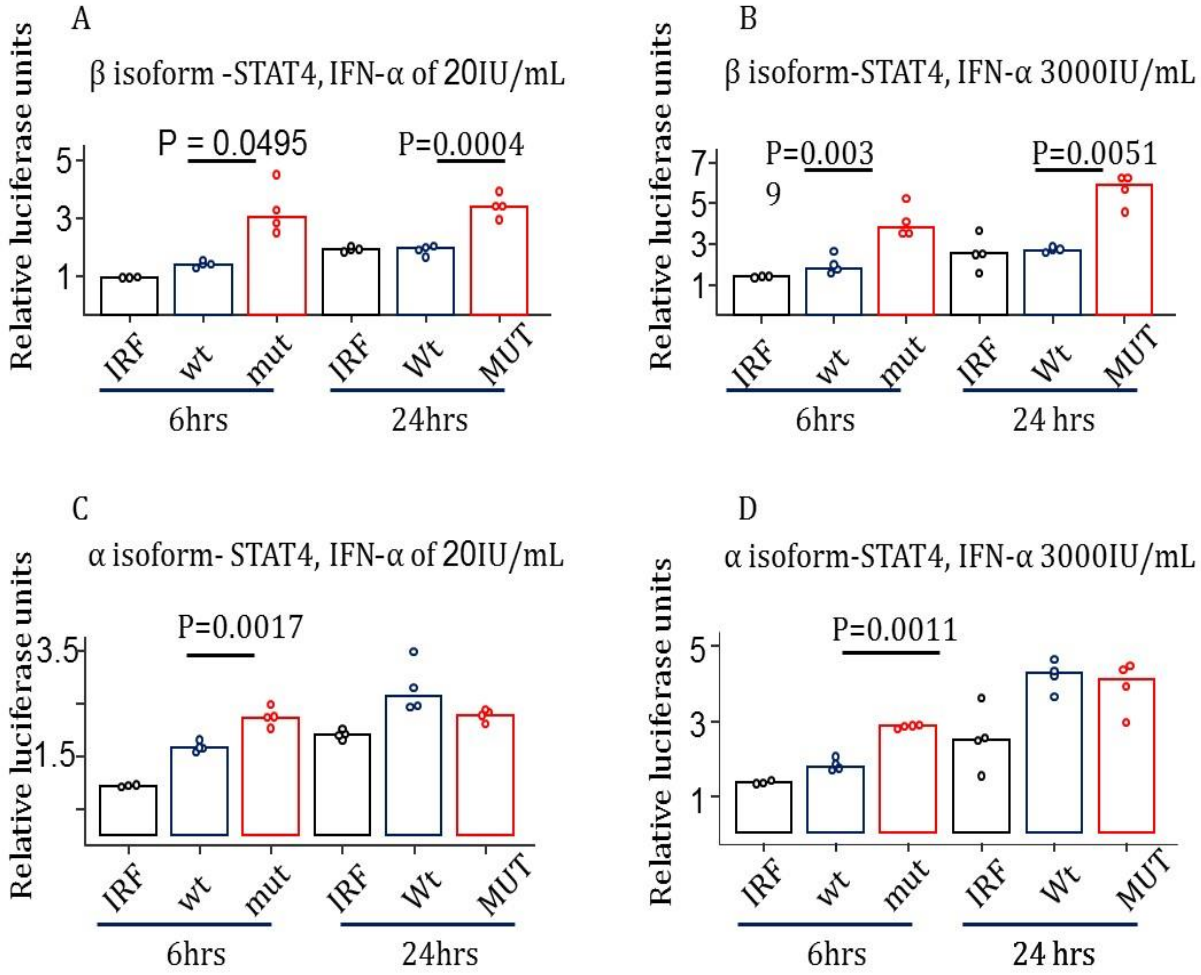

Figure 3-8 STAT4 P450S is associated with prolonged transcription

HEK293T cells were transfected with IRF1 (firefly signal), Cmv-PRL (renilla signal) and either nothing extra (IRF+Cmv-PRL only, black), wild type (wt, blue) or mutant (mut) of *STAT4* and subjected to luciferase assay according to the manufacture's recommendation (detailed in chapter 2.3.2.15). **A** β isoform of *STAT4* was used and stimulation was done using IFN-α (20IU/ml) for 6 hours or 24 hours**. B**. β isoform of *STAT4* was used and stimulation was done using IFN-α (3000IU/ml) for 6 hours or 24 hours**. C.** α isoform of *STAT4* was used and stimulation was done using IFN-α (20IU/ml) for 6 hours or 24 hours. **D.** α isoform of *STAT4* was used and stimulation was done using IFN-α (3000IU/ml) for 6 hours or 24 hours. The obtained firefly

signal was first normalized according to the renilla signal and then expressed as relative change compared to the unstimulated isoform. Independent sample t-test was used, $P<0.05$ significant.

Taken together, these results suggest a model of action in which $STAT4^{P450S}$, affects the DNA binding domain of *STAT4* and therefore protect STAT4 from the dephosphorylation by prolonged nuclear binding leading to enhanced transcription of IRF that leads to IFN-γ production.

3.7 IL-12-STAT4 system is important for T-bet up-regulation but not the only determinant

T-bet is the master transcriptional regulator of Th1 cells (Figure 3.5) (Szabo et al. 2000), and therefore, the proportion of cells expressing T-bet expression can be used as a measure of the extent of Th1 deviation. Naïve T cell do not express measurable level of T-bet. T-bet up-regulation can be a direct result of IL-12/ STAT4 activation but can also be induced indirectly by IFN-γ and STAT1 activation (Lighvani et al. 2001; Afkarian et al. 2002). FACS sorted naïve CD4+ T cells from a normal control were stimulated with TCR and co stimulation signals through the use of CD2/3/28 with or without IL-2 have induced T-bet expression (Szabo et al. 2000; Szabo et al. 2002), possibly through induction of IFN-γ that would in turn stimulate STAT1 leading to T-bet up regulation. The addition of IL-12 to the same system of TCR and co stimulation (CD2/3/28+IL-2) was necessary for optimal T-bet up-regulation (Figure 3.5F and Figure 3.9A) (Ylikoski et al. 2005; Ramos et al. 2007; Thieu et al. 2008).

Examination of T-bet in PBMCs revealed that almost all CD3+ T cells were T-bet positive in II.3 (Figure 3.9B). PBMCs from II.2, the sibling with $STAT4^{P450S}$ also exhibited much less impressive deviation to high level T-bet expression (Figure 3.9B). We then examined naïve CD4+T cells, which normally express negligible levels of T-bet (Szabo et al. 2000). At baseline, the level of T-bet in FACS sorted

naïve CD4+T cells from II.3, a control and other member of his family was low (Figure 3.9C). Thus, T-bet over-expression is only observed after T cell activation and the high level of T-bet in $STAT4^{P450S}$ is not due to simply abnormal high expression of T-bet that could be detected even in the naïve CD4+ T cells.

Since the IFN-γ–STAT1 pathway is also important for T-bet up-regulation (Afkarian et al. 2002; Lighvani et al. 2001), we went on to examine the possibility that high T-bet in the proband (II.3) is due to abnormal active STAT1. Therefore, we examined pSTAT1. Levels of STAT1 phosphorylation were similar in monocytes and lymphocytes from II.2 and II.3 and healthy donors (Figure 3.9 D-F). This finding argues against abnormal active STAT1 as the explanation for enhanced T- bet expression in II.3.

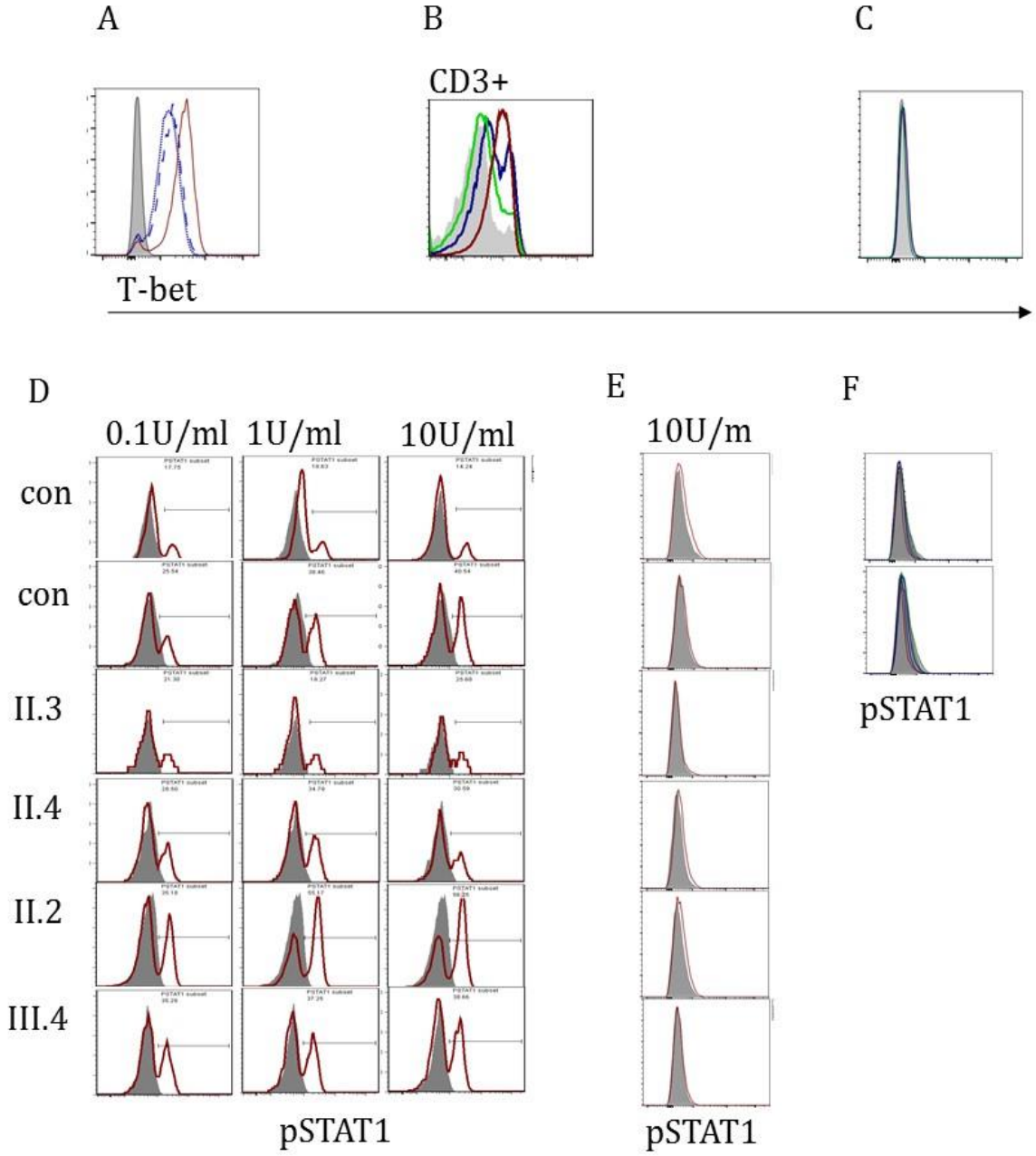

Figure 3-9 Exceptional T-bet expression

A. Overlay histogram of level of T- bet on FACS sorted naïve CD4+T cells from a normal control (filled grey), naïve CD4+T cells cultured for four days with TCR and co stimulation (CD2/3/28)(unfilled dotted blue), or with (CD2/3/28) and IL-2(unfilled blue long dashed, IL-2 (80ng/ml) or CD2/3/28 with +IL-2 (80ng/ml) +IL-12 (20ng/ml, red (unfilled red), fixed, permabilised and then stained for T-bet. **B.**

Overlay histogram of level of T-bet on CD3+. Control (filled grey), II.3 (red unfilled). II.2 (green unfilled) and II.4 (unfilled blue), PBMCs fixed, permabilised and then stained for T-bet. **C.** Overlay histogram of level of T-bet on FACS sorted naïve CD4+T cells. Control (filled grey), II.3 (red unfilled). II.2 (green unfilled) and II.4 (unfilled blue), fixed, permabilised and then stained for T-bet. **D.** Overlay histogram of level of pSTAT1 in PBMCS gated on monocytes and stimulated with different doses of IFN-γ (Filled grey is unstimulated versus stated dose of IFN-γ (red unfilled) for 20 minutes, fixed, permabilised and then stained for pSTAT1 in controls(n=2) and different members of the family. **E.** Overlay histogram of level of pSTAT1 in PBMCS gated on lymphocytes and stimulated with of IFN-γ (10U/ml), (Filled grey is unstimulated) versus IFN-γ stimulated (red unfilled) for 20 minutes, fixed, permabilised and then stained for pSTAT1. **F.** Overlay histogram of level of PSTAT1 in PBMCS gated on lymphocytes from the controls (n=2 filled, grey), II.3 (red unfilled), II.2 (green unfilled), and II.4 and III.4 (blue unfilled), all unstimulated (upper histogram) and all stimulated with IFN-γ(10U/ml) lower histogram.

3.8 STAT4 and TFH

TFH and cTFH are characterized by expression of CXCR5 and IL-21 production. Our understanding of the signals necessary for TFH and cTFH formation remains incomplete. In humans, IL-12 and STAT4 signalling gives rise to Th1 (Kaplan, Sun, et al. 1996; Robertson et al. 2005) subset but recently, IL-12 has also been implicated in TFH differentiation (Schmitt et al. 2009). Consistent with this suggestion, patients with IL12Rβ1 deficiency have significantly reduced number of cTFH marked by low number of circulating CXCR5 (Schmitt, Bustamante, and Bourdery 2013). These patients are also deficient in memory B cells.

In view of this evidence, we evaluated cTFH formation in the family members carrying *STAT4* gain-of-function mutation. The number of CD4+ T cells that had adopted cTFH phenotype (CXCR5+CD45RA-) phenotype in II.3 was found to be significantly higher than the normal controls. By contrast, II.2 had a normal

proportion of CXCR5+ CD45RA- CD4+ T cells raising the possibility that CXCR5 formation is not entirely dependent on STAT4 (Figure 3.10 A-B). Previously published results indicated that IL-12Rβ1 deficient patients had fewer CXCR5+ CD4+ T cells, and that the level of CXCR5 expression was lower compared to normal controls(Schmitt, Bustamante, and Bourdery 2013). We found no difference in median expression of CXCR5 in our two $STAT4^{P450S}$ cases compared to normal controls.

Next, we examined IL-21 and IFN-γ ex-vivo expression and induction. The percentage of ex vivo IFN-γ positive cells as shown previously was high for both II.2 and II.3 (Figure 3.2), though higher for II.3 compared to normal controls. The percentage of cells which were IL-21 positive cells was also high in both $STAT4^{P450S}$ siblings (II.2 and II.3) (Figure 3.10 C-D) compared to normal controls.

IL-12 signalling via STAT4 has also been implicated in the generation of TFH from naïve CD4+T cells. Therefore, we assessed induction of TFH (IL-21 production in cells co expressing TFH markers) from FACS sorted naive CD4+T cells cultured under IL-12 containing conditions and co-stained for either PD-1 or ICOS. Both the II.2 and II.3 exhibited an increase in IL-21+ CD4+ T cells that co-expressed high PD-1 or ICOS, compared to other controls (Figure 3.10 E-H). Of note, similar to the induction of Th1 in previous sections, induction of TFH from naïve CD4+ T cells in both II.2 and II.3 was cytokine-dependent, as there was no evidence of IL-21 induction in conditions that had no IL-12 (Figure 3.10 E-H).

BCL-6 is known as a master regulator for formation and maintenance TFH (Johnston et al. 2009; Nurieva et al. 2009). Previous work has suggested that BCL6 up-regulation in humans is IL-12 and STAT4 dependent (Nakayamada et al. 2011). Analysis of cells immediately ex-vivo revealed that there was no difference in the level of BCL-6 expression which was similar in PBMCs from both II.2 and II.3 and healthy donors (Figure 3.10 I). In mice, it has been shown that IL-12 and STAT4 can lead to BCL-6 up-regulation, which then leads to up-regulation of IFN-γ and T-bet,

which in turn suppresses expression of BCL-6 (Nakayamada et al. 2011). Next, we stimulated naïve T cells with CD2/3/28 beads and IL-12. This resulted in higher BCL-6 expression in naïve T cells from II.3 but not in II.2 (Figure 3.10 J).

Pathological actions of STAT4, MTOR and STAT3 in human T cell differentiation

Figure 3-10 STAT4P450S is associated with increase IL-21 producing TFH
A. Representative flow cytometry analysis of circulating TFH in propand (II.3) and a normal control. **B.** Summary of the frequency (%) of CXCR5+CD45RA- cTFH out of CD4 +T cell compartment in the propand (II.3), II.2 and other family members and normal controls(n=10). **C.** Representative flow cytometry analysis of intracellular ex-vivo IL -21 detection in CD4+T cells in II.3 and a normal control after 4 hours stimulation with PMA and ionomycin and brefeldin-A. **D.** Summary of the frequency (%) of IL-21 positive CD4+T cells in II.3, II.2 and normal controls (n=9). **E.** Flow cytometry analysis of frequency of intracellular IL-21 and PD-1 positive cells out of a system of FACS sorted naïve CD4+T cells cultured for 4 days with IL-12 (20ng/ml) +CD2/3/28 +IL-2(80ng/ml) in a control and II.3 .**F**. The same analysis was done on FACS sorted naïve CD4+ T cells of the same control and II.3 cultured for four days with CD2/3/28 and IL-4 (20 µg /ml). **G.** Flow cytometry analysis of frequency of intracellular IL-21 and ICOS positive cells out of a system of FACS sorted naïve CD4+T cells cultured for 4 days with IL-12 (20ng/ml) +CD2/3/28 +IL-2(80ng/ml) in a control and II.2 **H.** The same analysis was done on cells obtained from FACS sorted naïve CD4+ T cells cultured in the presence of CD2/3/28 and IL-2 (80ng/ml) only**. I.** Histogram of the level of BCL-6 of freshly thawed PMCs, obtained from (II.2 (green unfilled), II.3 (red unfilled), II.4 (blue unfilled) and a normal control (grey filled).**J.** Histogram of the level of BCL-6 induction in FACS sorted naïve CD4+ T cells cultured with low dose of IL-12(1ng/ml) +CD2/3/28.(II.2(green unfilled),II.3(red unfilled),II.4(blue unfilled) and a normal control(grey filled).

In summary, $STAT4^{P450S}$ was associated with an enhanced TFH formation, assessed by ex-vivo IL-21 production. This was more evident in the in-vitro induced system with IL-21 production and co-expression of other TFH markers such as ICOS and PD-1.

3.9 B cell phenotype in the presence of Novel STAT4P450S and expanded Th1 and cTFH

STAT4 is important for IFN-γ production by T lymphocytes but STAT4 has negligible expression in B cells. Assessment of ex-vivo IFN-γ production by B cells of the available family members did not yield any evidence of IFN- γ over production by the carriers of $STAT4^{P450S}$ (II.2 and II.3) (Figure 3.11 A). Moreover, STAT4 is important for IL-21 producing TFH formation and this subset is essential for B cells function and plasma cell formation (Schmitt, Bustamante, and Bourdery 2013). II.3 exhibited an increased number of CXCR5 cTFH, and both II.3 and II.2 exhibit increased expression of IL-21 in CD4+ T cells ex-vivo. Despite this enhanced TFH activity, the B cell phenotype of II.3 revealed reduced B memory (CD19/CD27) with normal transitional B cells (Figure 3.1 A). By contrast, the B cell phenotype of II.2 was normal for memory and transitional cells (Figure 3.6 C). In other words, despite the expanded TFH assessed by CXCR5 and ex vivo production of IL-21, there was no expansion of memory B cells in II.3. On the other hand, as noted above, before initiation of IVIG replacement, II.3 had high total IgA and IgG, which was mainly made up of IgG1 and IgG3, while IgG2 was reduced. He mounted a suboptimal specific antibody to tetanus toxoid. By contrast, II.2 had a normal immunoglobulin profile with normal subclass and specific antibody responses.

Assessment of plasma cells exvivo in II.2 revealed a slight increase compared with controls ($CD19^{high}$ CD38+ $CD27^{high}$) (Figure 3.11. B).This has prompted us to examine B cell responses to activation *in vitro*. B cells from both II.2 and II.3. PBMCs from both II.2 and II.3($STAT4^{P450}$) as well as other controls, were stimulated for four days with CpG and IL-21 alone, or in the presence of either IFN-γ or anti IFN-γ. This was done to assess the ability of B cell to form plasma cells to investigate the possibility that IFN-γ might be inhibitory for plasma cell formation in the presence of this novel mutation. Cells from controls as well as both II.2 and II.3 showed a normal ability to form plasma cells (Figure 3.11 C). In fact, overall addition of IFN-γ did not impair plasma cell formation in all samples especially in the

condition containing CpG (Figure 3.11C-D). Thus, the presence of IFN-γ *in vitro* experiment had a positive effect on plasma cell formation. Assessment of *in vitro* class switching revealed enhanced IgG1 and IgG3 formation in B cells from II.3 (Figure 3.11E-F). Moreover, II.3 had ex-vivo propensity toward IgG1 and IgG3 deviation and this was replicated in the *n-vitro* system (Figure 3.11E-F).

Pathological actions of STAT4, MTOR and STAT3 in human T cell differentiation

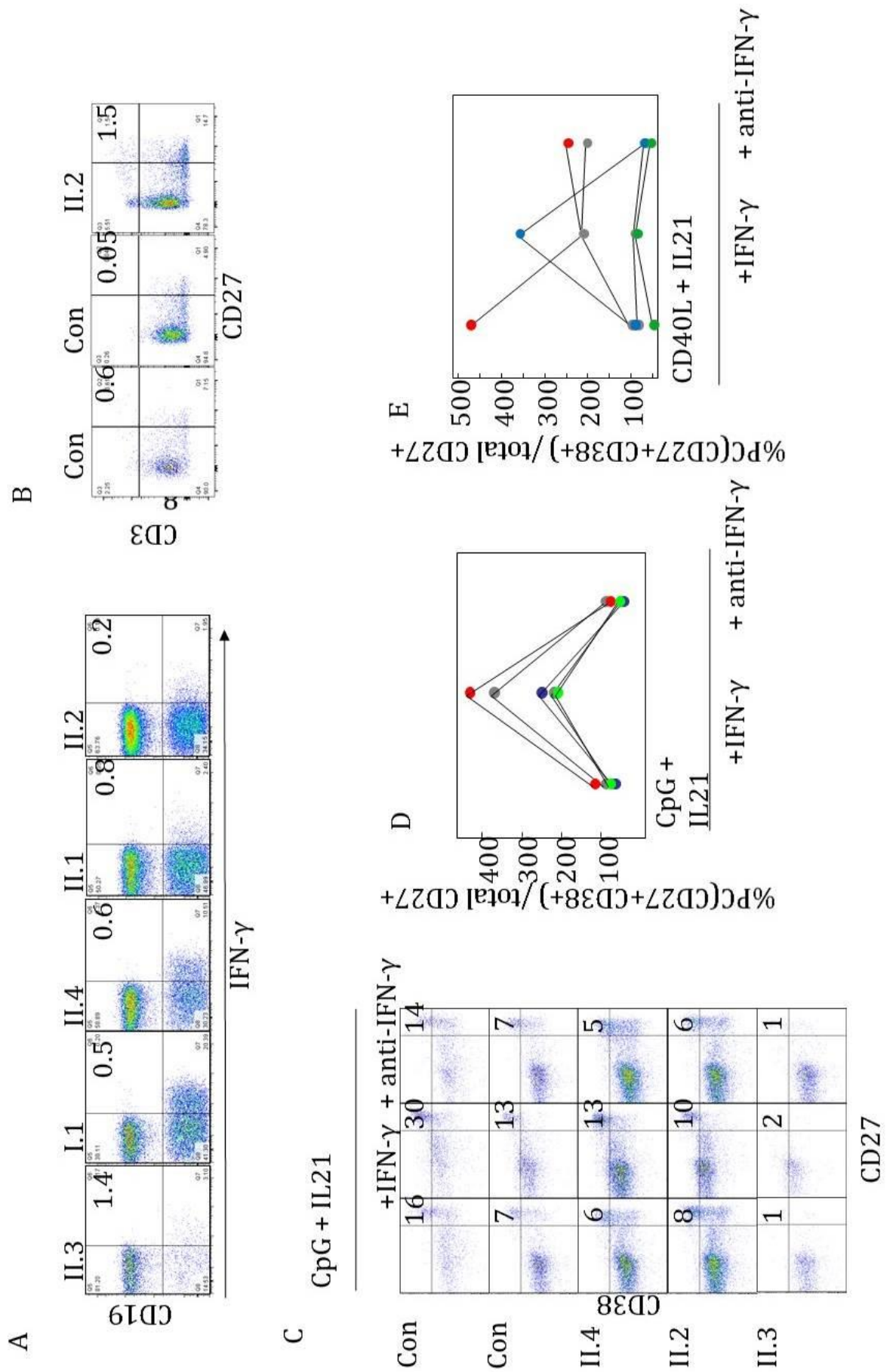

Figure 3-11 *STAT4* ^{P450S} and B cell phenotype

A. Flow cytometric analysis of ex-vivo intracellular IFN-γ by B cells from the available family members after 4 hours stimulation with PMA and ionomycin and brefeldin-A .**B.** Circulating plasma cells (gated as CD27 ^{high} and CD38 ^{high}) in II.2 and normal controls (n=2).. **C.** Flow cytometry analysis of induction of plasma cell after stimulation of PBMCs with a stimulus that is either CPG(1uM) +IL-21(10ng/ml) or CD40L(1 µg)+IL-21(10ng/ml) with either (IFN), IFN-γ (100U/ml) or (anti) anti-IFN-γ (I µg /ml) for four days. PBMCs were washed, stained for 7AAD, CD3, CD14 (negative gate), then gated on CD19, CD27. PBMCs obtained from available family members (n=3) and normal controls (n=2). **D.** Summary of the frequency (%) of induced plasma cell as proportion of the memory B cells **E.** Induction of subclass switching in II.3 and normal controls (n=2) after stimulating PBMCs with CD40L (1 µg /ml) and IL-21(10ng/ml) for four days. PBMCs were then washed, surfaced stained for CD19, CD27&CD38 and then fixed, permabilised and then stained for IgG total and subclass. Gates were set using gating on a freshly thawed and stained tonsil sample. **F.** Summary of the frequency (%) of different IgG subclass out of induced plasma cell (II.3), and normal controls (n=2).*,$p<0.05$

3.10 Chapter summary and discussion

We discovered a novel and private germ line *STAT4*^{P450S} in a discovery project utilizing second generation sequencing (whole exome sequencing) in a proband (II.3) recruited to the Australian and New Zealand Antibody Deficiency Allele (ANZADA) project. We have shown that the same mutation was found in another otherwise healthy sibling (II.2) and one of her offspring.

STATs are made of very similar domains and mutations affecting different domains have been described to impede or enhance development of specific effector T cells. *STAT4*^{P450S} is a novel mutation affecting DNA binding domain of *STAT4*. Mutations in the DNA binding domain have recently been discovered in association with a gain

of function in general. STAT1 gain of function due to mutations in the DNA binding domain has been seen in patients with different clinical phenotypes ranging from CMC (Takezaki et al. 2012; Yamazaki et al. 2014), HLH (Faitelson et al. 2014) ,invasive dimorphic yeast infection (Sampaio et al. 2013) and IPEX like clinical phenotype (Uzel et al. 2013). All of these germline mutations of the DNA binding domain gain of function were associated with increased activation and phosphorylation of STAT1 in response to cytokine stimulation and subsequent increased transactivation of its target genes (Frans et al. 2014; Takezaki et al. 2012; Yamazaki et al. 2014; Sampaio et al. 2013). Recently, germline mutations in the DNA binding domain of *STAT3* have also been reported in patients with solid organ autoimmunity and early onset lymphoproliferation (Milner et al. 2015). These mutations were also associated with high STAT3 activation and phosphorylation in response to activating cytokines (Milner et al. 2015). Therefore, based on association of most DBD mutations with a gain of function phenotype, we have assumed that this mutation of *STAT4* is a gain of function (GoF). Up to our knowledge there are no reported cases of *STAT4* GoF mutations. However, polymorphism possibly being a gain of function was reported to be associated with increased risk of autoimmunity and inflammatory conditions (Pang et al. 2007; Remmers et al. 2007; Martinez et al. 2008; Sigurdsson et al. 2008; Zervou et al. 2008).

STAT4 deficiency is associated with lack of Th1 development in mice. Furthermore, it is known that IL-12-STAT4 system is crucial in up regulation of T-bet, master regulation of formation and maintenance of Th1. Therefore , IL12, STAT4, T-bet system is very important for adoption and maintenance of Th1 phenotype in the CD4+ and CD8+ T cells (Figure 3.5 A-B). Mice deficient in either IL-12, STAT4 or T-bet have defective Th1 formation which can be assessed by measuring Th1 signature cytokine and that is IFN-γ (Kaplan, Sun, et al. 1996; Cho et al. 1996; Nishikomori et al. 2002; Szabo et al. 2002; Szabo et al. 2000; Ylikoski et al. 2005; Hsieh, Macatonia, Tripp, Wolf, Ogarra, et al. 1993; Magram et al. 1996).Our results have shown excessive Th1 deviation assessed by presence of abundance of ex-vivo IFN-γ in the CD4+ and CD8+ T cells compartment in II.3 (proband)(Figure 3.2 A-D)

as well as in II.2 though slightly to a lesser extent(Figure 3.6 E). These finding of exceptional Th1 phenotype were replicated by the in-vitro culture system in which naïve (unpolarised cells) were separated and polarized to become Th1 versus other lineages. Cells were cultured under Th1 polarizing ((CD2/3/28) +IL-12), and this combination supports the expression and activation of STAT4 protein in the cells. This has resulted in detection of almost twice the amount of the IFN-γ (Figure 3.2 H upper row) compared to the amount detected in cells came from a normal control. Moreover, the Th1 formation even in the carrier of $STAT4^{P450S}$ was accentuated and was also cytokine dependent (Figure 3.2 H lower row). This was more evident after examining cells harbouring $STAT4^{P450S}$ in a system that has lacked either STAT4 expression or activation as in the conditions of Th2 polarization (CD2/3/28 +IL-4), Figure 3.6 E upper row) or neutral conditions as in (Th0(CD2/3/28 +IL2), Figure 3.6 E lower row) of STAT4. Therefore, so far we have established that $STAT4^{P450S}$ is associated with deviated Th1 phenotype in the T cell compartment in human in line with literatures from animal models.

$STAT4^{P450S}$ is a novel mutation involving the highly conserved DNA binding domain in *STAT4*. STAT4 is activated after stimulation with IFN-α or IL-12. Phosphorylated STATs in general bind to their elements in the nucleus via the DNA binding domain (Xu, Sun, and Hoey 1996; Meyer et al. 2003; Horvath, Wen, and Darnell 1995) and be protected from dephosphorylation (Mertens et al. 2006; Meyer et al. 2003). The longer they bind to these elements the longer the effect of trans-activating specific genes. The level of STAT4 in lymphocytes is low in resting state (Bacon, Petricoin, et al. 1995) and any stimulation with either IFN-α or IL-12 did not lead to any stimulation of STAT4 (Figure 3.5 C). However, after stimulating cells with agents such as PHA or TCR and co stimulation using (CD2/3/28) (Bacon, Petricoin, et al. 1995) , assessment of STAT4 can be done. In polarized Th1 conditions, cells carrying $STAT4^{P450S}$ have demonstrated a prolonged and sustained phosphorylation compared to other controls (Figure 3.7 A). Moreover, we attempted to replicate the same finding using transfected HEK293T cell line with mutant allele versus wild allele. The mutant allele has demonstrated a prolonged phosphorylation of STAT4 assessed

by flow-cytometry and western blot compared to the wild type after stimulation (Figure 3.7 B-D). In addition, STAT4 binds to the *IRF1* element in the nucleus upon its activation and phosphorylation. Therefore, examination of the nuclear localization and the *IRF1* transactivation can be used as readout to assess the gain of function of $STAT4^{P450S}$. The direct immunofluorescence of transfected HEK293T cells with wild type versus mutant type post stimulation with IFN- α has revealed a nuclear localization in the mutant but not in the wild allele transfected cells (Figure 3.7 E). Moreover, examination of the IRF1 signal in cells transfected with either mutant or wild allele of *STAT4* has shown clearly a prolonged transcription of *IRF1* (Figure 3.8 A-D).

All of the above finding supported the initial assumption that $STAT4^{P450S}$ is a gain of function mutation. To our knowledge there is no reported gain of function mutation in human mutation affecting DNA binding domain or any other domain in STAT4. $STAT4^{P450S}$ is a cytokine dependent and was associated with prolonged phosphorylation (Meyer et al. 2003). $STAT4^{P450S}$ upon stimulation resulted in an enhanced transcriptional activation of its target genes shown by the luciferase reporter. In the reported literatures of Th1 deficiency in mice through defects in the STAT4 signalling pathway, Th1 deficiency was associated with deviated Th2 formation(Kaplan, Sun, et al. 1996; Usui et al. 2003) (Wood et al. 2005). In human, however, there was no enhanced Th2 formation in patients with different defects in the IL-12 IFN-γ pathway (Wood et al. 2005). Similarly, our data demonstrated that expanded Th1 due to STAT4 did not affect frequency of Th2 CD4+T cells. This might suggest that in human *STAT4* does not cross regulate *STAT6* or other Th2 related genes directly or indirectly in contrast to the *STAT1-STAT3* cross regulation (Takezaki et al. 2012; Yamazaki et al. 2014; Liu et al. 2011). However, this possibility would need to be examined in details.

Persistent STAT4 leads to T- bet up regulation, both of which are needed for Th1 formation in the CD4+ T and CD8+ T compartments (Figure 3.5 A-F) (Yamamoto et al. 1994; Zhong, Wen, and Darnell 1994; Kaplan, Sun, et al. 1996).

The described *STAT4* mutation here presented with significant Th1 deviation and T-bet up regulation (Figure 3.9 B).The same finding was substantiated using cells from the affected sibling with the same mutation. The presence of high T-bet is associated with Th1 response (IFN-γ) associated with IgG1 predominant immune response (Stoicov et al. 2009).

Finally, STAT4 has been recently linked to the formation of TFH cells. We observed enhanced IL-21 expression in cTFH cells within the proband, II.3, and indeed in his $STAT4^{P450S}$ sibling, II.2, consistent with enhanced Il-21 expression as a result of *STAT4* gain of function (Figure 3.10). This was also replicated in the in-vitro system of inducing TFH using IL-12 as a stimulus (Figure 3.10-E-H). Our findings provides the first *in vivo* evidence for the importance of STAT4 for human Th1 (IFN-γ), T-bet up regulation and TFH formation.

Pathological actions of STAT4, MTOR and STAT3 in human T cell differentiation

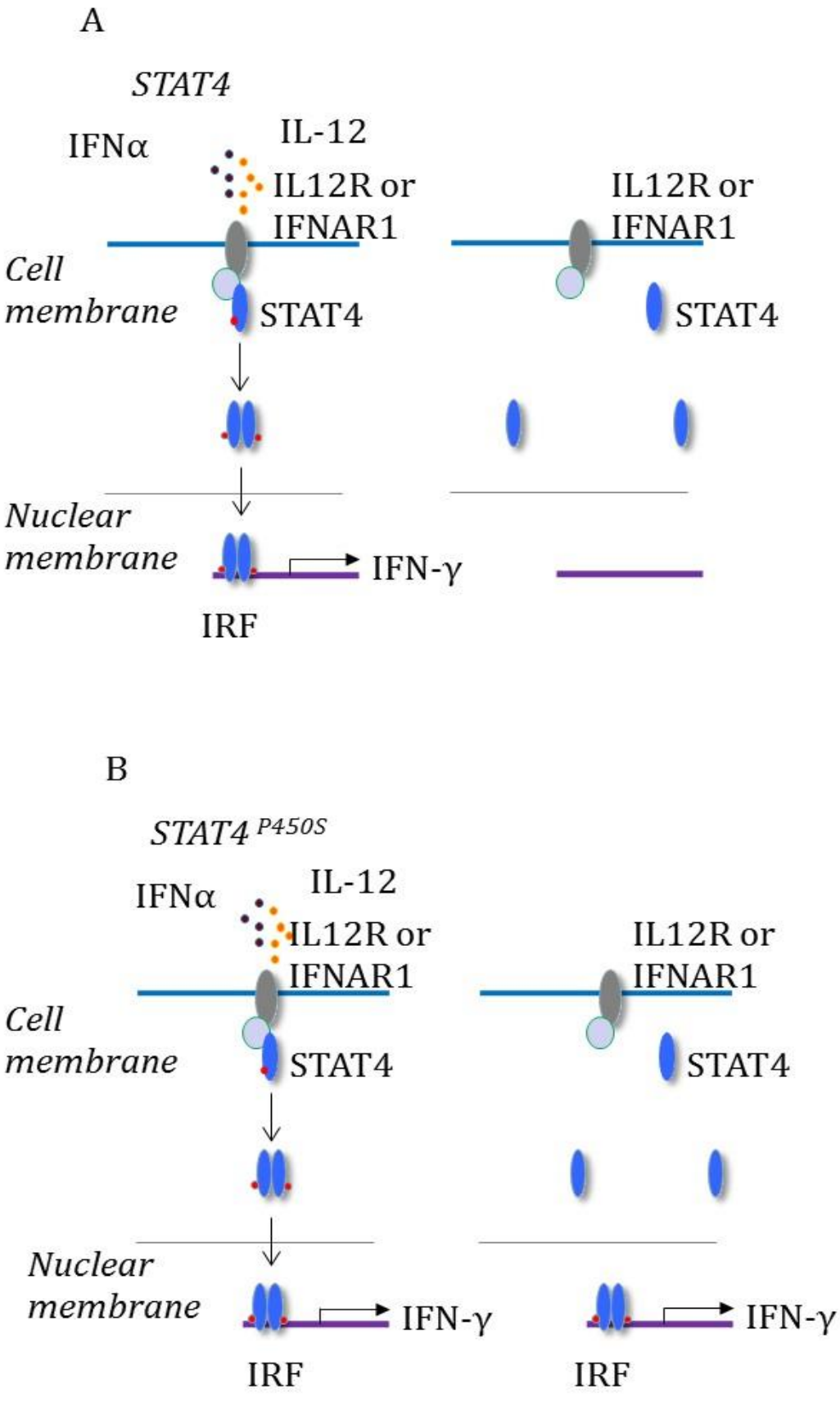

Figure 3-12 Proposed model of action of *STAT4* ^{P450S}

A. Wild allele of *STAT4* is stimulated with either IFN-α or IL-12 which leads to phosphorylation of STAT4. Phosphorylated STAT4 forms dimer, is recruited to the nucleus to bind to its transcription region *(IRF1*). In cells bearing wild allele of *STAT4* , pSTAT4 is dephosphorylated and leave the nucleus**. B.** However, in the cells bearing *STAT4*^{P450S} , pSTAT4 is bound to the nucleus for a longer time and therefore exert a prolonged transactivation of its target genes(*IRF1*). Moreover, its binding to the nucleus protect against dephosphorylation.

CHAPTER 4 : *MTOR* ^{T2448M} IS A NOVEL GAIN OF FUNCTION ALLELE

4.1 Introduction

On comparison of CD4 +and CD8+ T cells from II.2 and II.3 for the extent of Th1 deviation (assessed by IFN-γ) and T-bet levels in CD3+ T cells, we identified a trend towards Th1 deviation which was less marked in II.2 compared to II.3 despite the fact that both were carriers of the *STAT4*^{P450S} GoF mutation. Therefore, this raised the possibility that the exceptional Th1 phenotype in II.3 compared to II.2 might not be all due to *STAT4*^{P450S}.

Moreover, we have shown that II.3 has marked effector CD8+ T cell phenotype with expansion of Temra subset. We therefore considered the possibility that the extreme effector Th1 phenotype observed in II.3 is the result of a modifier mutation.

4.2 Exceptional effector Th1 CD8+ and CD4+ T cells phenotype is a result of STAT4 GoF and a modifier mutation

Pathological actions of STAT4, MTOR and STAT3 in human T cell differentiation

As shown earlier in the previous chapter, the CD8+ T cell compartment in II.3 exhibits prominent expansion of the Temra subset compared to normal adult controls (Figure 3.1 C-D) in addition to the remarkable Th1 phenotype (Figure 3.2). Examining the rest of the available family member for these two signatures revealed that there are members who expressed a similar phenotype though slightly to a lesser extent of Th1 deviation in CD4 +T cell and CD8+ T cell as well as expansion of the Temra subset in CD8+ T cells (Figure 4.1 A-D, in blue). Therefore, there are other members in the family whom exhibited these two main findings of Th1 deviation (IFN- γ) and expansion of terminally differentiated CD8+ T cell.

Pathological actions of STAT4, MTOR and STAT3 in human T cell differentiation

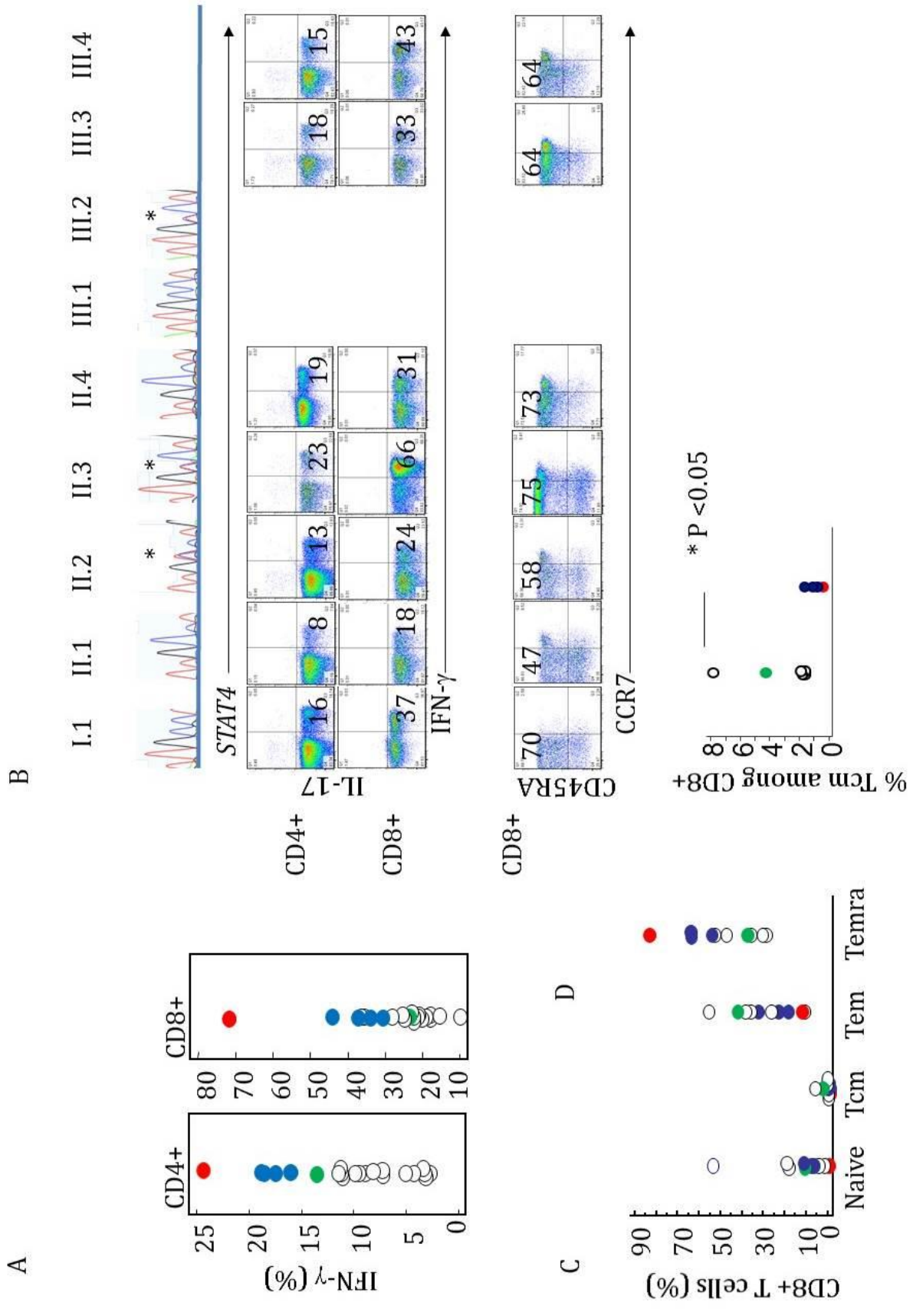

Figure 4-1 Exceptional Th1 CD8+ and CD4+ T cell phenotype is a result of *STAT4* GoF and a modifier mutation

A. Summary of % of intracellular IFN-γ producing CD4+ and CD8+ T cells obtained by flow cytometry after 4 hours stimulation with PMA and ionomycin and brefeldin-A in controls(n=16, unfilled circles), II.3(red filled circle, heterozygous $STAT4^{P450S}$) II.2 (green filled circle) and I.I, II.4, III.3 and III.4 (blue filled circles, heterozygous $STAT4^{P450S}$). B. Electropherogram of *STAT4* in the available family members depicting the heterozygous $STAT4^{P450S}$ *(1st row from top, star),* intracellular IL-17 versus IFN-γ expression in CD4+ and CD8+ T cells (*middle row*) obtained by flow cytometry after 4 hours stimulation with PMA and ionomycin and brefeldin-A in different family members. CD8+ subsets analysed according to CD45RA and CCR7 expression in the available family members (*lower row*). Available family members, (starred, II.2, II.3 and III.2, heterozygous $STAT4^{P450S}$), (un starred I.1, II.4, III.3 and III.4, wild type *STAT4*). .C .Summary of % of different circulating CD8 T subsets naive (CD45RA+CCR7+), Tcm (CD45RA-CCR7+), Tem (CD45RA-CCR7-) and Temra(CD45RA+CCR7-). D summary % of Tcm (significance was calculated using Man-Whitney test) obtained by flow cytometry in controls (black unfilled, n=5) and available family members (red filled II.3, heterozygous $STAT4^{P450S}$), (green filled II.2), (blue filled, I.1, II.4, III.3 and III.4, heterozygous $STAT4^{P450S}$).

4.3 CD8+ T cell subsets and effect of mTOR CD8 subsets

CD8+ T cells can be divided into four different subsets according to their surface markers (CD45RA and CCR7) CCR7 (Hamann et al. 1997; Sallusto et al. 1999; Tomiyama et al. 2004). These subsets are functionally different and exhibit different cytokine profiles (Hamann et al. 1997; Sallusto et al. 1999). CD45RA+CCR7+ naïve T cells have negligible cytotoxic ability or IFN- γ production. On the other hand, CCR7- subsets, both CD45RA+ and CD45RA-, are effector memory cells that are

both cytotoxic and IFN- γ producers. CD45RA- CCR7+ T cells have no immediate effector or cytotoxic activity, but these cells are easily induced in a recall response. In young subjects, most CD8+ T cells are naïve. With aging, memory effector CD8+ T cells predominate in the circulation and Tcm CD8+ T cells predominate in lymphoid tissue(Koch et al. 2008).

Recent evidence indicated that blocking mTOR does not only inhibit immune system activation but has also paradoxical action that fine tunes CD8+T cell immunity. mTOR signalling through the mTORC1 complex influences memory versus effector differentiation of naïve CD8+ T cells (Araki et al. 2009). Blocking mTOR with rapamycin promotes formation of memory CD8+ T cells (Tcm) (Araki et al. 2009), (Rao et al. 2010) and (Ramos et al. 2009). These effects appear to be CD8+ T cell-intrinsic, since RNA interference of mTOR solely in CD8+ T cells replicates the effect of incubating PBMCs with rapamycin (Araki et al. 2009). Furthermore, the use of mutant CD8+ T cells, rendered specifically insensitive to rapamycin failed to replicate the phenotype of expanded memory CD8 T cells when cells were treated with rapamycin (Araki et al. 2009).

4.4 Segregation of CD8+ T cell phenotype and the variant of MTOR

We reanalysed the whole exome sequence from II.3 for candidate modifiers. We identified a novel heterozygous missense mutation in (Figure 4.2 A-B) in *MTOR* (*MTOR*^{T2446M}). The mutation encoding *MTOR*^{T2446M} is unique and predicted to be damaging by in silico analysis. This amino acid substitution is located in the negative regulatory domain of *MTOR*. Sanger sequencing confirmed the mutation in II.3 and other family members (I.1, II.4, III.3 and III.4) (Figure 4.2 C-D). Of note, the finding that the family members exhibiting expansion of effector Th1(IFN-γ) and Temra CD8+ T cell subset (Figure 4.1 A-D, in blue) are the same members carrying this novel variant of *MTOR* (*MTOR*^{T2446M}).Thus, the mutation segregates with this cellular phenotype. Of considerable interest, II.3 is the only member of the kindred to carry mutations in both *STAT4* and *MTOR* (Figure 4.2 D).

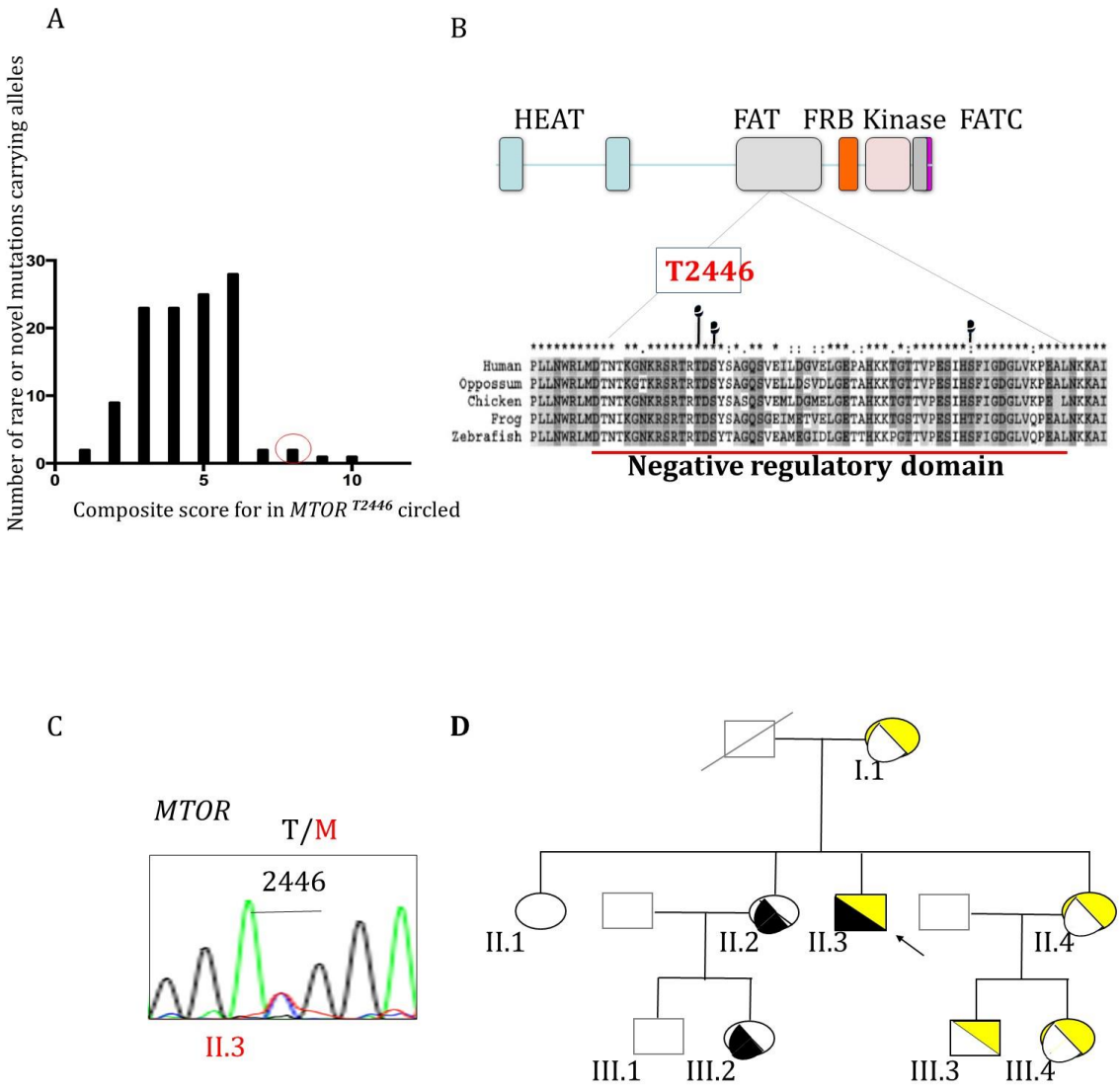

Figure 4-1 Segregation of TEMRA expansion phenotype and *MTOR*^{T2446M}

A. Filter Score obtained for novel missense mutation (*MTOR*) score circled. **B.** Protein structure including T2446 in the negative regulatory domain (upper row). Alignment of NRD of different species (lower row). **C.** Electropherogram depicting the heterozygous 2446T>M mutation in the patient (II.3). **D**. Family tree. Heterozygous *STAT4* ^{P450S} in black and heterozygous *MTOR* ^{T2446M} in yellow.

4.5 MTOR T2446M is a not a loss of function mutation

Inhibition (Brunn et al. 1997) or deletion (Sekulic et al. 2000b) of the highly conserved negative regulatory domain (NRD) leads to constitutive activity of mTOR. The NRD has 3 phosphorylation sites, T2446, S2448 and S2481. Serine 2448 is phosphorylated by protein kinase B (PKB) after stimulation by insulin (Sekulic et al. 2000a). On the other hand, the phosphorylation of Ser2448 is attenuated with amino acid deprivation. In contrast, nutrient deprivation activates AMP activated protein kinase (AMPK), which results in phosphorylation of Thr2446. This is thought to reduce mTOR activity, reflected in diminution of P70S kinase activity (Cheng et al. 2004). Interestingly, P70S kinase itself has the ability also to phosphorylate both T2446 and S2448. (Holz and Blenis 2005). There is some evidence to suggest that phosphorylation of T2446 and S2448 might be a mechanism of mutual cross-regulation, which integrates availability of nutrition and growth factors (Cheng et al. 2004) .

Moreover, as described above (Chapter 3.5 and 3.7), rapamycin leads to expansion of Tcm (opposite to the phenotype observed in Figure 4.1 C-D) and reduction in effector CD8+ T cells Tem in the circulation and secondary lymphoid organs, and inhibits formation of tissue memory CD8+ T cells. On the other hand, active mTOR is needed for formation of effector subsets of CD8+ T cells (Araki et al. 2009; Rao et al. 2010; Rao, Li, and Shrikant 2010) through up regulation of T-bet , master regulator of effector CD8+T cells. The T-bet up regulation and effector phenotype is impaired in the presence rapamycin that favour up regulation of eomesodermin, master regulator for central memory phenotype. We have demonstrated previously more deviated effector CD8 +T cell phenotype and Th1 (IFN-γ) (Figure 4.1).

Taken together, the biochemical importance of T2446 and the negative regulatory domain and the known action of mTOR on CD8+ T cell differentiation toward effector CD8+ T cell differentiation, this lend plausibility to the postulate that

T2446M in *MTOR* is a functional mutation toward effector Th1 phenotype and most likely to be a gain of function mutation.

Therefore, we proceeded to examine the functional consequences of the $MTOR^{T2446M}$ mutation. We first tested the opposite possibility to our assumptions, that $MTOR^{T2446M}$ confers a reduction in mTOR function rather than a gain of function. First, we examined the effect of addition of rapamycin to CD8+ T cells from healthy donors. T cells were stimulated with CD2/3/28 and rapamycin was added in the presence or absence of IL-12 for 3 days. We observed an increase in percentage of cells adopting Tcm phenotype (CCR7+CD45RA-) and a significant reduction in the number of cells adopting the effector phenotype (Tem and Temra) (Figure 4.3A-C).

Next, we performed the same experiments using cells isolated from II.2 ($STAT4^{P450S}$ and wild type *MTOR*). If the $MTOR^{T2446M}$ mutation is a loss of function (LoF) then we might expect to recapitulate the phenotype observed in II.3 of expanded extreme Temra over other subsets. Furthermore, treatment of rapamycin did not lead to increase in the total number of CD8+ T cells. (Figure 4.3A-C).

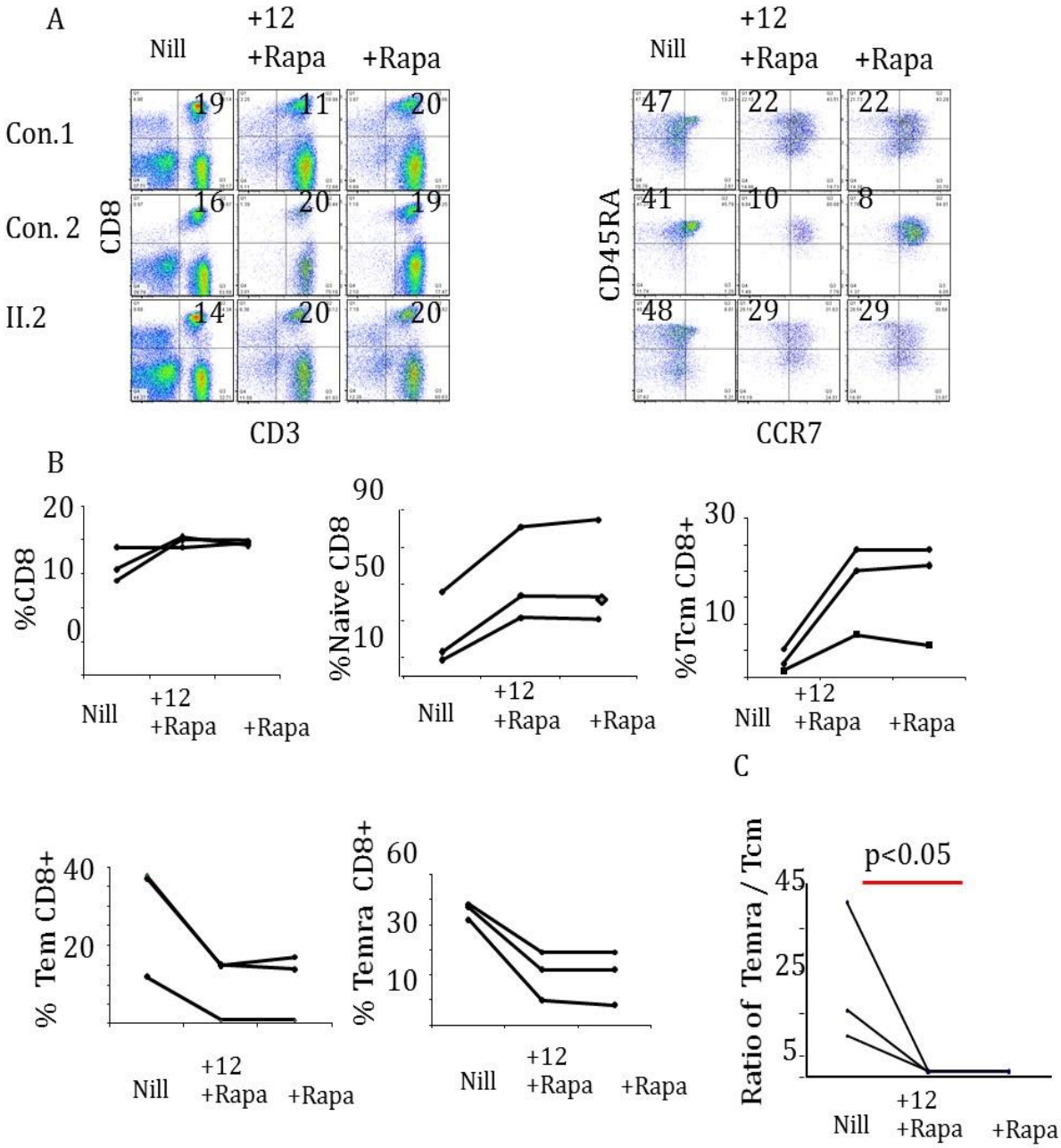

Figure 4-2 Active mTOR is important for the formation and maintenance of Temra and Tem CD8Tcells

A. Flow cytometry analysis of surface markers of CD 8 and its subsets in controls(n=2) and II.2 in freshly thawed PBMCs (Nill) , after 3 days culture with

rapamycin (100ng/ml) with IL-12(2ng/ml) (+Rapa+12) or Rapamycin alone (+Rapa)**B.** Summary plots of the percentage of total and different subsets of CD8 in the three different conditions (controls in grey and II.2 in green). **C**. Ratio of Temra over Tcm obtained for each of the controls and II.2 in the three different conditions ($p<0.05$, Friedman test).

Similarly, treatment of CD4+ T cells with rapamycin led to more cells adopting naïve and Tcm surface phenotype rather than effector phenotype (Figure 4.4 A-C). Taken together, these findings suggested that if the $MTOR^{T2446M}$ mutation is functional and it is likely to confer a gain of function (by reducing activity of the negative regulatory domain) rather than being a loss of function.

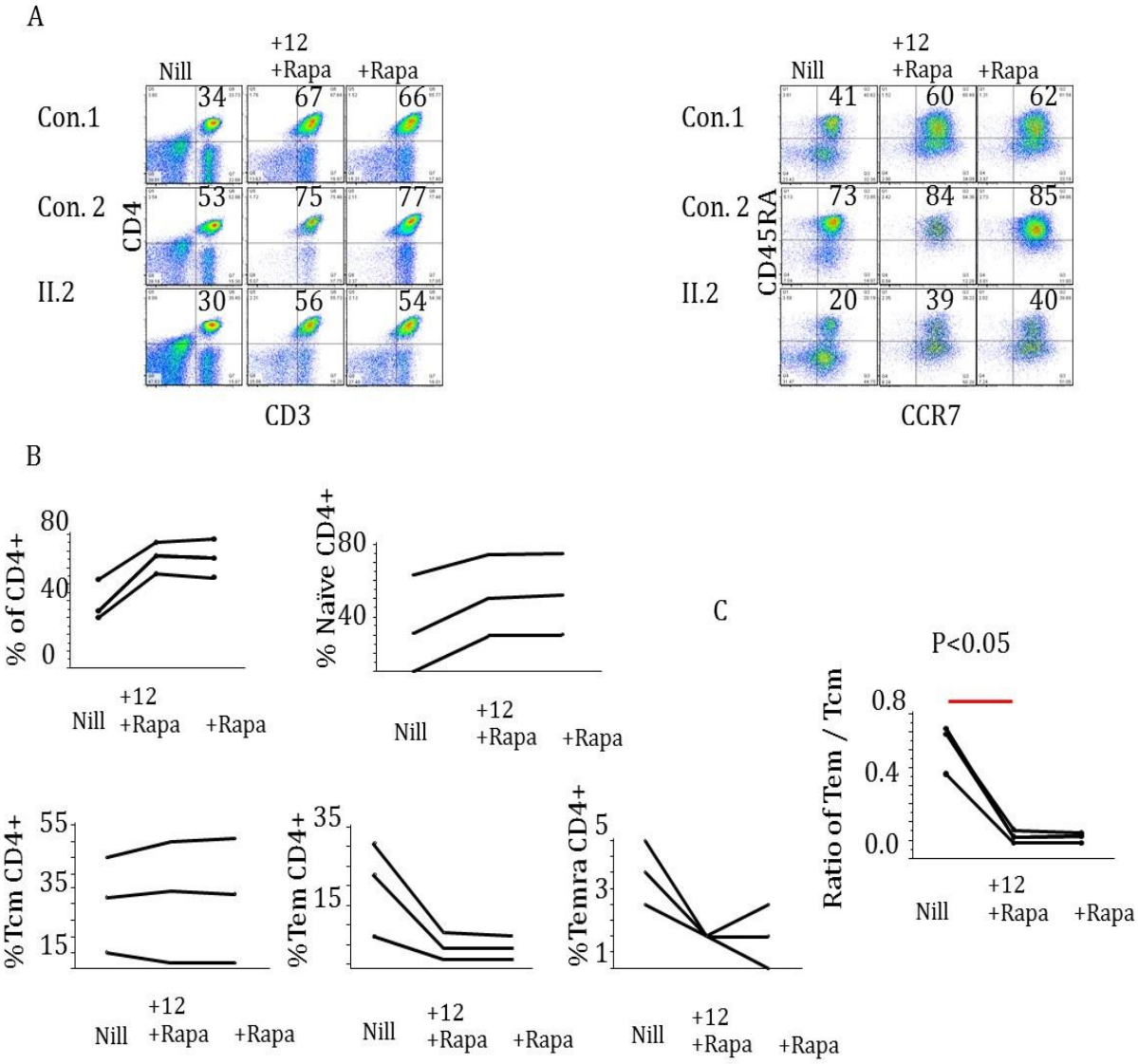

Figure 4-3 Active mTOR is important for the formation and maintenance of Temra and Tem CD4Tcells

A Flow cytometry analysis of surface markers of CD4+ T subsets in controls and II.2 as in freshly thawed PBMCs(Nill) , after 3 days culture with rapamycin (100ng/ml) with IL-12(2ng/ml) (+Rapa+12) or Rapamycin alone (+Rapa)**B.** Percentage of different subsets of CD4+ T cells in the three different conditions in controls (n=2) and II.2. **C.** Ratio of Tem over Tcm obtained for each of the controls and II.2 in the three different conditions. ($p<0.05$, Friedman test).

Pathological actions of STAT4, MTOR and STAT3 in human T cell differentiation

IL-7 is necessary for proliferation and homeostasis of naïve CD8+ T cells (Tan et al. 2001) as well as homeostasis of resting memory T cells (Schluns et al. 2000; Goldrath et al. 2002). IL-7Rα (CD127) is thought to promote CD8+ T cell survival as IL-7R expressing naïve and Tcm express the highest percentage of BCL-2 as anti-apoptotic marker (Araki et al. 2009), and cells deficient in IL-7R are more prone to apoptosis in mice (Schluns et al. 2000). Both naïve and Tcm memory CD8+ T cells express higher levels of CD127 than effector subsets (Tem and Temra) or recently activated CD8+ T cells (Schluns et al. 2000).

Active mTOR signalling leads to down regulation of CD127, which is thought to promote formation of effector CD8+ T cells, and IFN-γ and granzyme- B production. As stated earlier that rapamycin (blocking mTOR) enhances transition of Tem CD8+ T cells into Tcm CD8+T cells (Araki et al. 2009) but as well it lead to persistent expression of CD127 (Rao et al. 2010). Therefore, we proceeded to examine the effect of the $MTOR^{T2446M}$ on the level of CD127 surface expression on different CD8+T cells and other effector phenotype such as level of expression of granzyme-B in normal versus our proband (II.3).

We examined IL-7 receptor α (CD127) expression in CD8+ T cells from a healthy control. In keeping with published findings, we found that Tcm and naïve CD8+ T cells express higher levels of IL-7R α (CD127) and minimal granzyme- B. By contrast, Tem and Temra have low expression of CD127 and are enriched for IFN-γ and granzyme-B production (Figure 4.5 A and C). We performed the same comprehensive phenotyping of CD8+ T cells from (II.3). This revealed a relative increase in IFN-γ and granzyme- B expression in the Temra subset with low CD127 expression (which constitutes the major CD8+ T cell subset in II.3) (Figure 4.5 B and D). We examined the ratio of Temra to Tcm CD8+ T(Araki et al. 2009) cells from other family members. This revealed a trend toward increased effector CD8+ T cells relative to Tcm in the members carrying the *MTOR* ^{T2446M} mutation (Figure 4.5 E) with enrichment for IFN-γ and granzyme-B(GzmB) production (Figure 4.5 F). The

above findings are consistent with the postulate that *MTOR* ^{T2446M} mutation is a gain of function leading to effector Th1 phenotype in CD4+ T but also in CD8T+ cells.

Pathological actions of STAT4, MTOR and STAT3 in human T cell differentiation

Figure 4-4 Effector CD8+ T cell phenotype due to *MTOR*^{T2446M}

A-B. Representative flow cytometry analysis of level of IL-7Rα, CD127 expression by different CD8+T subsets and their production of IFN-γ in a control (**A**) and in II.3 (**B**). **C-D.** Representative flow cytometry analysis of level of granzyme-B expression by different CD8+ T cell subsets in a control (**C**) and in II.3 (**D**). **H.** Summary of ratio of percentage of Temra over Tem controls (n=5) and *MTOR*^{T2446M} (n=3). The difference was $p<0.05$ using wilcoxon test for independent samples. **I.** Summary of percentage of granzyme-B positive CD8+ T cells from normal controls (unfilled black circles), II.2 (filled green), II.4 (filled blue) and II.3 (filled red).

4.6 MTORT2446M is a gain of function mutation

So far, the shown functional cellular data supported our initial postulate that *MTOR*^{T2446M} confers gain of function. In order to support this postulate further, we proceeded to biochemical evaluation of the mTOR pathway. S6 kinase is a direct substrate of mTOR. Cellular activation via the mTOR pathway results in phosphorylation of 70 S6 Kinase (pS6K) and subsequent S6 phosphorylation (pS6) (Brown et al. 1995). The activation of mTOR can be achieved either through the use of PMA (Oubrahim et al. 2013) or TCR engagement (Hamilton et al. 2014). Therefore, PBMCs from family members and controls were either left unstimulated or stimulated with PMA, and were then lysed and analysed by western blot for the level of pS6K. Three individuals with a *MTOR*^{T2446M} genotype showed higher baseline phospho-S6K1 compared to normal controls, supportive of a gain-of-function or constitutively active allele (Figure 4.6 A-B). Activated S6K catalyses phosphorylation of S6. We therefore also examined pS6 by flow cytometry in controls and three of four family members identified with *MTOR*^{M2446T} and similarly demonstrated enhanced pS6 at baseline as well as after stimulation. This effect was reversed by co-culture with rapamycin (Figure 4.6 C). These findings are consistent with the *MTOR*^{M2446T} mutation conferring gain of function on mTOR. This is most likely the result of loss of function of the negative regulatory domain of *MTOR*. This

is in keeping with previous data that deletion(Sekulic et al. 2000a) or blocking (Brunn et al. 1997)of this domain give rise to active mTOR .The consequence of this mutation is effector Th1 phenotype deviation especially with a deviation toward Temra (Figure 4.6D).

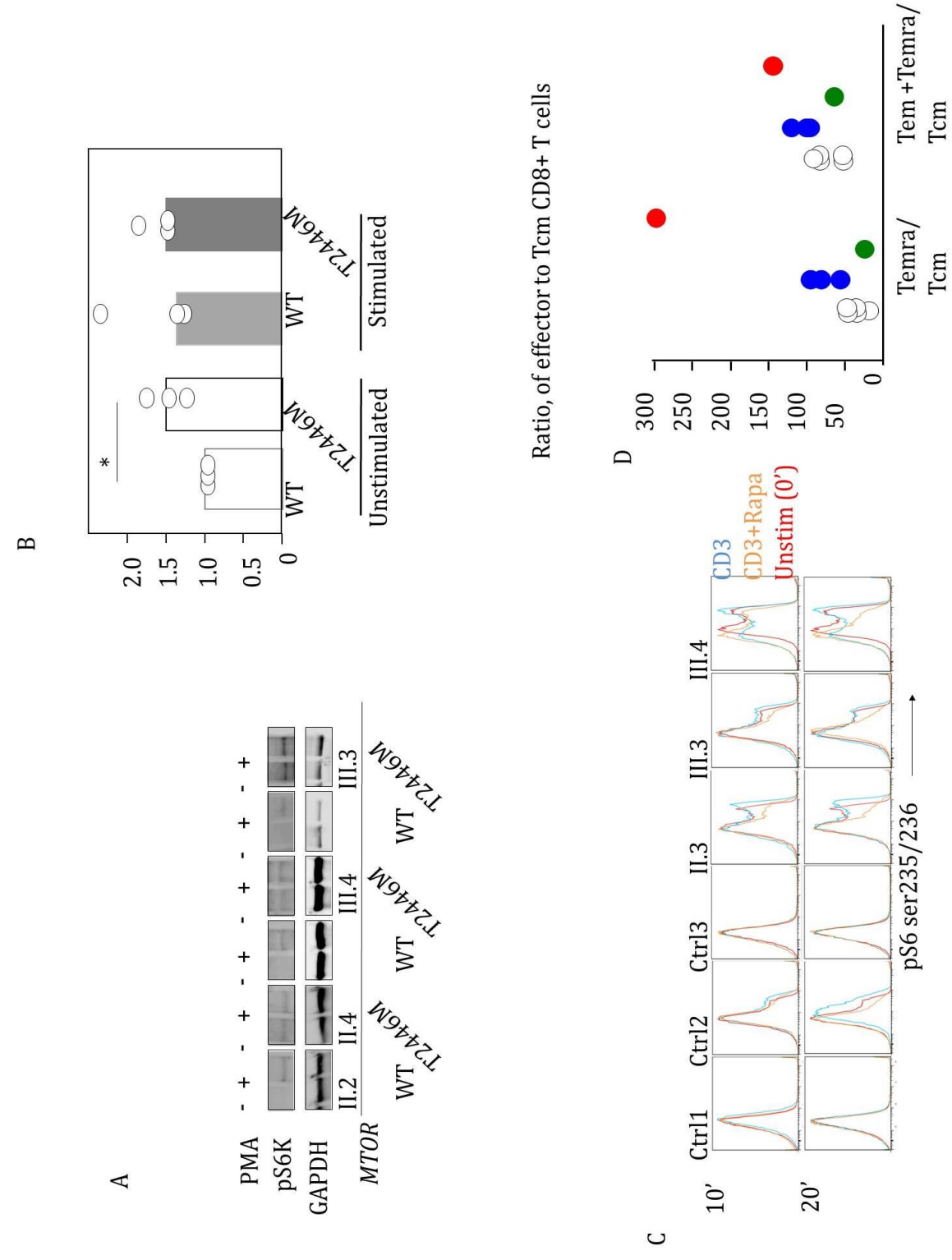

Figure 4-5 *MTOR*^{M2446T} is a gain of function mutation

A. Western blots of pS6K. PBMCS were either not stimulated or stimulated with PMA (50nM) for 30 minutes, lysed or the lysate was then run on Tris glycine minigel from 3 normal controls and II.4, III.3, III.4. **B.** Summary of the normalised pS6 against GAPDH (housekeeping protein) in the unstimulated conditions and the PMA stimulation. P<.05, using Wilcoxon test for independent samples. **C.** Histogram of level of pS6 (ser235/236) in activated T cells at 0, 10 and 20 minutes after being unstimulated (red unfilled histogram), stimulated with CD3 (unfilled blue) or with CD3 and rapamycin (unfilled orange). **D.** Summary of the ratio of Temra over Tcm or effector (Temra and Tem) over Tcm CD8+ T cells from in different donors. Normal controls (n=5 unfilled black circle), II.4, III.3, III.4 (filled blue), II.2 (filled green) and II.3 (filled red).

4.7 Interaction between MTOR and STAT4

4.7.1 Introduction

Activation of naïve CD8+ T cells by IL-12 acting via STAT4 (signal 3) is thought to be crucial for normal CD8+ T cell differentiation. IL-12 induces the effector phenotype of CD8+ T cells, including IFN-γ, perforin, granzyme-B as well as cytolytic activity of CD8+T cells(Ramos et al. 2009)and enhances up-regulation of T-bet, which in turn regulates formation of Tem over Tcm with high expression of T-bet in Tem compared to Tcm(Ramos et al. 2009).

As we have just shown in previous sections (figure 4.3-4.6), there is considerable evidence that mTOR regulates effector cell differentiation of CD8+ T cells (Araki et al. 2009; Rao, Li, and Shrikant 2010; Rao et al. 2010). Active mTOR supports T-bet up regulation, IFN-γ and granzyme-B production (Rao, Li, and Shrikant 2010; Rao et al. 2010).

In fact, we find mTOR is a plausible positive modifier for STAT4 due to many reasons. Activation of STAT4 by IL-12 enhances mTOR activation, when compared with antigenic stimulation alone (Rao et al. 2010). Conversely, mTOR signalling

system is necessary to maintain active STAT4 signalling. In addition based on previous literatures, both IL-12 and mTOR are crucial for the expansion of effector T cells especially in the CD8+ compartment, through up regulation of T-bet and down regulation of eomesodermin.

Taken together the facts that, there is a biochemical importance of T2446 , the known action of mTOR on CD8+ T cell differentiation and the importance of both IL-12STAT4 and active mTOR toward effector CD8+ T cell differentiation, lend plausibility to the postulate that *MTOR* ^{T2446M} is positive a modifier of *STAT4*^{P450S} both of which acting toward exaggerated Th1 responses, and this what we aimed to investigate next.

4.7.2 STAT4 and MTOR cooperate toward maximal effector CD8+ T cell and CD4+ T cell differentiation giving rise to high T-bet signature

To investigate the effect of STAT4 activation by IL-12 on activation of mTOR, we first cultured naïve CD4+T cell isolated from a normal donor with CD2/3/28 and IL-2 for 4 days with or without IL-12. TCR and co stimulation (CD2/3/28) has caused activation of mTOR assessed by pS6. The addition of addition of IL-12 to this experimental system has caused optimal activation of mTOR (pS6) (Figure 4.7A). Therefore, the activation of STAT4 was needed to induce maximal activation of mTOR. In addition, in the same system, stimulation of the cells with TCR and co stimulation (CD2/3/28) has up regulated the level of T-bet. However, IL-12 resulted in maximal T-bet up-regulation (Figure 4.7B).

In other words, activation of TCR and co stimulation (CD2/3/28) has activated mTOR and up regulated T-bet but maximal activation of both, mTOR and up regulation of T-bet is IL-12 and STAT4 dependent. These experiments support previous observations that IL-12 acting via STAT4 activates mTOR and up regulates T-bet (Rao et al. 2010; Li et al. 2011).

Next, to show that active mTOR is required to up regulate T-bet and maintain active STAT4, we treated PBMCs from a normal control with CD2/3/28 and IL-12 with and without rapamycin. TCR and co stimulation (CD2/3/28) in the presence of IL-12 resulted in maximal up regulation of T-bet, and this was sensitive to rapamycin (Figure 4.7C). Moreover, phosphorylation of STAT4 also appears to be mTOR sensitive, as rapamycin reduced the extent of STAT4 phosphorylation observed in II.3 to the same level seen in normal controls (Figure 4.7 D). These experiments support the observation that active mTOR is required for optimal STAT4 activation and T-bet up regulation. The experiments shown here points toward that both active STAT4 and active mTOR are needed for maximal T- bet expression.

Approximately 20% of all human CD4+ T cells express T-bet. Approximately 60% of all human CD8+ T cells express T-bet, with Temra and Tem exhibiting the highest levels of expression (Joshi et al. 2007). The proportion of T-bet positive cells is highest in the effector memory compartment (Knox et al. 2014). Therefore, the level of T-bet expression can be taken as readout for the extent of Th1 deviation in CD4+ and CD8+ T cells.

We evaluated T-bet (*TBX21*) expression in the proband (II.3) and available family members. Un-manipulated PBMCs taken from the proband (II.3) were uniformly T-bet positive (Fig. 3.9B). The proportion of T-bet+ cells were increased in II.2 $STAT4^{P450S}$ single mutant CD3+ T cells, but not to the same level observed in the II.3. II.4, $MTOR^{T2446M}$, single mutant CD3+ T cells analysed immediately ex vivo also exhibited increased T-bet expression which was even higher than we observed in II.2 (Figure 4.7 E). Taken together, these findings are consistent with constitutively active allele of $MTOR^{T2446M}$ mutation that enhances T-bet expression. However, since T-bet requires activated STAT4 for Th1 differentiation, these effects remain subclinical unless combined with another abnormality, in this case, cytokine dependent overactive $STAT4^{P450S}$ both together leading to high T-bet expression and so effector CD4+ and CD8+ T cell differentiation. All of this would explain the pathological

elevation of T-bet even at the level of mucosa when assessed on gut biopsy (Figure 4.7 F).

Pathological actions of STAT4, MTOR and STAT3 in human T cell differentiation

Figure 4-6 *STAT4*^{P450S} *MTOR*^{T2446M} cross regulate each other toward extreme Th1 deviation

A. Overlay histogram of the level of pS6 (mTOR) in naïve CD4+T cells stimulated for 4 days with CD2/3/28 (grey shadowed histogram), CD2/3/28 with IL-2 (80ng/ml, blue unfilled) or CD2/3/28 +IL-2+IL-12 (20ng/ml, red unfilled). **B.** Overlay histogram of the level of T-bet in naïve CD4+T cells stimulated for 4 days with CD2/3/28 (grey filled histogram), TCR with IL-2 (80ng/ml ,blue unfilled) or CD2/3/28 +IL-2+IL-12 (20ng/ml ,red unfilled). **C.** Overlay histogram of the level of T-bet in CD3+T cells in freshly thawed PBMCs (grey filled histogram), stimulated for 4 days with (CD2/3/28), IL-12(20ng/ml) without (red unfilled) or with rapamycin (100ng/m, blue unfilled). **D.** Overlay histogram of the level of pSTAT4 in FACS sorted naïve CD4+ T cell stimulated for 3 days with TCR(CD2/3/28) and IL-12(20ng/ml) with and without rapamycin(100ng/m) in a control (grey filled histogram) and in II.3(red unfilled). **E.** Summary of the percentage of CD3+T-bet +analysed by flow cytometry in controls (n=6, filled grey), II.3 (red filled), II.2 (green filled) and II.4 (filled blue). **F.** Level of T-bet mRNA of tissue expression detected by RT-PCR on a biopsy obtained from the oesophagus. II.3 (red filled circle), controls (n=2 grey filled circles). G. Proposed model of interaction between STAT4 and mTOR toward extreme Th1 deviation.Stimulation of *STAT4*, leads to phosphorylation of STAT4 which is essential for T-bet upregulation and adoption of Th1 phenotype. Moreover, phosphorylated STAT4 activate MTOR complex especially mTORC1 . Active mTOR in turn activates STAT4 further. It also causes upregulation of T-bet in T cell that dictate the adoption of IFN-g Th1 producing phenotype. In cells bearing *STAT4*^{P450S} and *MTOR*^{T2446M} , the T-bet upregulation is accentuated giving rise to exceptional Th1 CD4+ and CD8 effector memory phenotype.

4.7.3 CHAPTER SUMMARY AND DISCUSSION

Active STAT4 and mTOR together drive T cell differentiation toward exaggerated Th1 deviation marked by IFN-γ production and T-bet up regulation. The proband (II.3) is the only one among his family to carry both *STAT4*^{P450S} and *MTOR*^{T2446M}. We have shown that *STAT4*^{P450S} is a gain of function mutation in the DBD causing

prolonged binding to the nucleus and therefore prolonger transcription of Th1 related genes.

MTOR^{T2446M} is a mutation in the highly conserved negative regulatory domain, the dysfunction of which was associated with constitutive mTOR as assessed by level of pS6. So far there is no reported human germ line mutation in mTOR though there are reports (Angulo et al. 2013; Lucas et al. 2014) of *PI3KCD* mutations that were shown to cause downstream activation of mTOR. The clinical and cellular feature in this cohort is similar to our proband and carrier of *MTOR*^{T2446M} in terms of expansion of CD8+ T cell effector subsets (Temra) and IFN-γ production.

Trm CD8+ T cells are a newly described subset that resides in non-lymphoid parenchyma. Trm appear to be more closely related to effector T cells, and have been implicated in inflammatory pathology such as in allergic contact dermatitis, psoriasis and crohn's disease. In contrast to the positive effect of blocking mTOR on CD8+ Tcell central memory abundance in SLO, rapamycin appears to inhibit Trm recruitment and formation.

T-bet up regulation occurs shortly after TCR ligation to some extent and is augmented and prolonged with activation of STAT4 through IL-12 (Szabo et al. 2000; Yang, Ochando, et al. 2007). Cells deficient in STAT4 failed to induce T-bet in mice (White et al. 2001). IL-12 STAT4 system is known for its positive effect on mTOR activation leading to effector T cell differentiation and T-bet up regulation. The unique combination of *STAT4* and *MTOR* gain of function mutations in the proband (II.3) can plausibly explain the extreme T-bet up regulation by all peripheral as well as tissue lymphocytes. The presence of high T-bet is associated with Th1 response (IFN-γ) associated with IgG1 predominant immune response (Stoicov et al. 2009) and some of the inflammatory diseases such as in the gastrointestinal tract (Kolumam et al. 2005). Up- regulation of T- bet is a main mechanism through which effector Th1 inflammatory response predominate in patients with crohn's disease (Matsuoka et al. 2004) gastritis and gastric cancer (Stoicov et al. 2009)as well as

acute graft versus host disease. Therefore, animals with deficiency in T- bet had less gut and liver GVHD (Fu et al. 2015). Moreover, patients with chronic GVHD (D'Asaro et al. 2006) had high representation of effector Temra CD8+ T cell. Higher T-bet was also responsible for inflammatory gut due to helicobacter felis leading to gastric cancer (Stoicov et al. 2009). On the other hand damping this high T-bet Th1 effector signature through administration of rapamycin treated dendritic cells was associated with prolonged cardiac allograft survival (Chiang et al. 2004). All the presented evidences support the possibility that extreme Th1 deviation and high T-bet expression might be responsible for the inflammatory phenotype exhibited by our patient's mucosal tissues.

CHAPTER 5 : DEPENDENCY OF IL-10 PRODUCTION BY HUMAN EFFECTOR CD4+ T TH2 CELL SUBSETS ON STAT3

5.1 Introduction

Autosomal dominant Hyper IgE syndrome (AD-HIES) is an immunodeficiency disease due to impaired function of STAT3 and subsequent failure in the formation of Th17 (Minegishi et al. 2007; Ma et al. 2008). However, one of the main criteria to diagnose AD-HIES is a prominent Th2-type phenotype, shared by other similar diseases mainly atopic eczema (Eyerich and Novak 2013), including high eosinophil count, high IgE levels and rashes (Schimke et al. 2010). While susceptibility to infection in this syndrome has been explained by the discovery that *STAT3* mutation compromises formation of TH17 cells, the explanation for high IgE in this disease remains unclear.

IL-10 is a potent regulatory cytokines against excessive Th1 and or Th2 effector immune response. This was made clear in animal models where reports have supported a role for STAT3 to induce IL-10 production in the context of chronic and excessive Th2 immunopathology (Altin, Goodnow, and Cook 2012). This STAT3

dependent production was by Th2 CD4+ T cells that remained Foxp-3 negative despite their new role as regulatory T cell.

It remains a possibility that there is a similar Th2 subset exists in human and the formation of this inducible subset is STAT3 dependent. Therefore we aimed to investigate requirement of STAT3 for normal IL-10 production by CD4+T cells and especially by Th2 CD4+ T cell making use of cells from patient with abnormal STAT3 signalling versus normal controls.

5.2 IL-10 positive human CD4+ T cells are rare and not STAT3 dependent

First, we compared the proportion of IL-10 producing CD4+ T cells isolated from normal controls and patients with AD-HIES. IL-10 producing CD4+ T cells are rare, accounting for less than 1% of CD4+ T cells in healthy donors (Figure 5.1 A-B). There was no difference between the frequency of these cells in normal controls and patients with AD-HIES. Moreover, in line with previous observations, we observed a higher proportion of IL-10+ cells in the memory compartment (CD4+ CD45RA-) compared to naïve (CD4+ CD45RA+) (Figure 5.1 C). These data suggest that baseline production of IL-10 from memory CD4+ T cells is not STAT3-dependent.

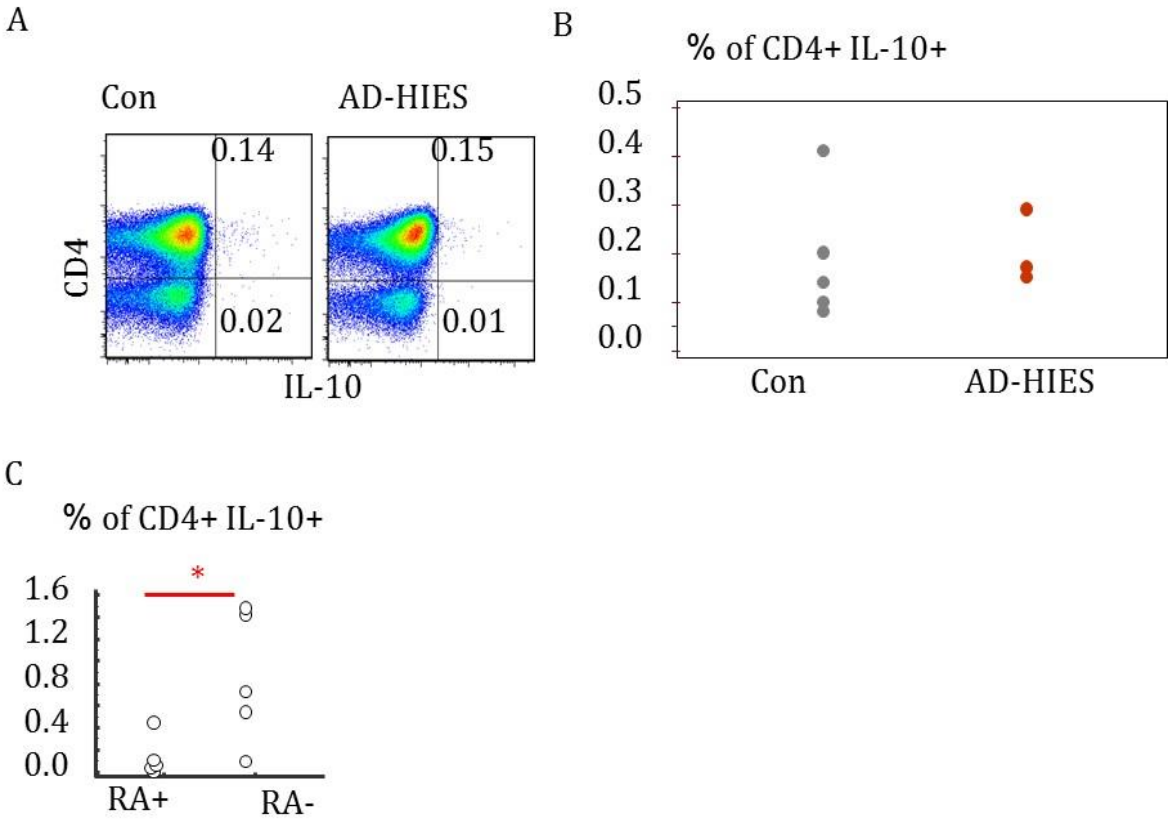

Figure 5-1 Ex vivo IL-10 positive human T cells are rare, not STAT3 dependent and mainly produced by memory CD4+T cells

A. Representative flow cytometry analysis of intracellular IL-10 expression by CD4+ T cells (CD3+CD4+) cells in a normal control(Con) and patient with ADHIES after 4 hours stimulation with PMA+ ionomycin and brefeldin-A. **B.** Summary % of IL-10 production by CD4+ T cells. ADHIES (red filled circle) and controls, n=5 (grey filled circles). **C.** Summary % of IL-10 production by RA+(CD45RA+) naïve and RA-(CD45RA-) memory CD4+ T cells in controls (n=5). Mann -Whitney test P <0.05.

5.3 IL-10 can be induced in CD4+ T cells in a STAT3-dependent way

Although we did not detect any significant difference in the proportion of ex vivo IL-10-positive CD4+ T cells between the two groups (Figure 5.1A-B), we proceeded to investigate the possibility that STAT3 might contribute to induction of IL-10 after

activation. PBMCs from a normal control and a patient with AD-HIES were stimulated with IL-21 in the presence of CD2/3/28 for 2 days. The ability to phosphorylate STAT3 in response to IL-21 is retained in patients with AD-HIES, as the syndrome usually results from missense mutations that result in amino acid substitutions in the DNA binding domain (Figure 5.2 A-B) or the SH2 domain. There was a significant induction of IL-10 in controls, which was not seen in the AD-HIES cells (Figure 5.2 C-D).

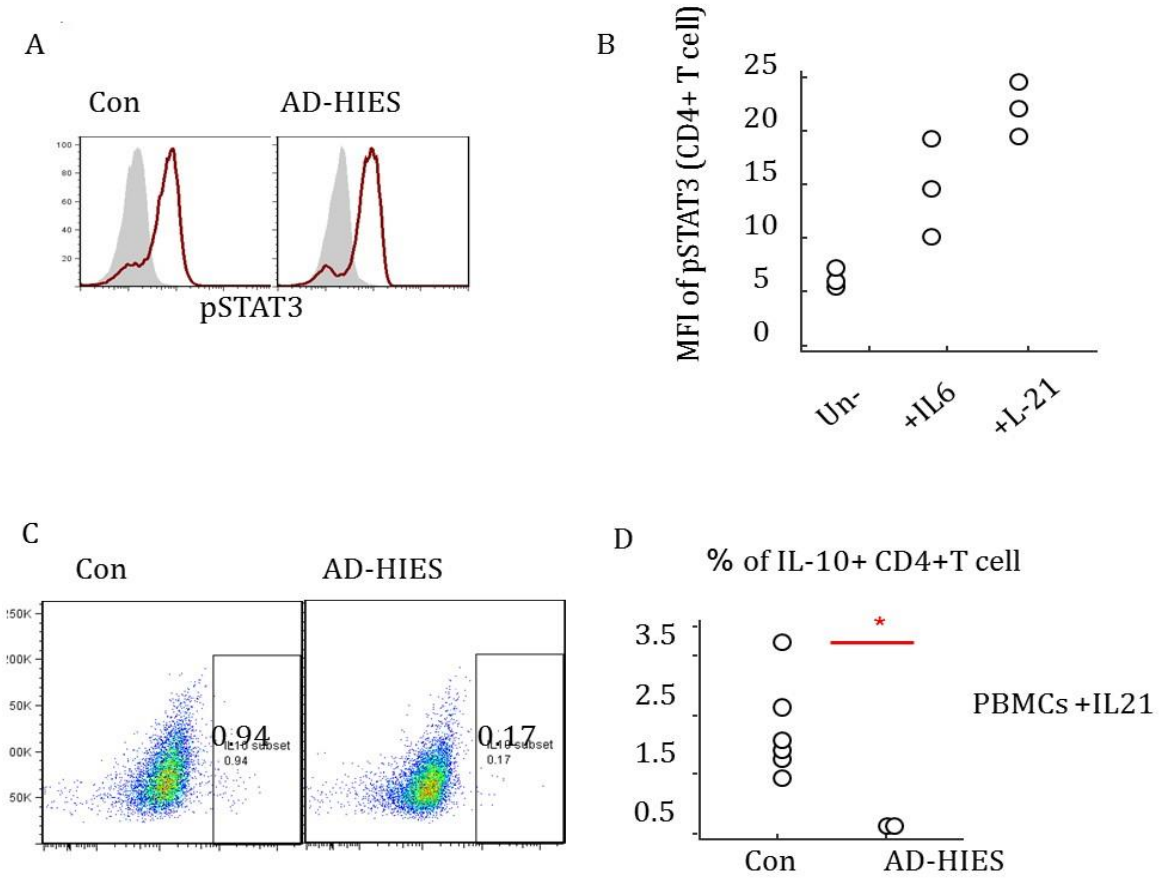

Figure 5-2 IL-21 can induce STAT3 dependent IL-10 production from human CD4+ T cells

A. Representative histogram of the level of pSTAT3 in a control and a patient with AD-HIES before and 20 minutes after stimulation of IL-21(100ng/ml), (unstimulated, grey filled. Stimulated, red unfilled). **B.** Summary of mean fluorescence intensity

(MFI) of pSTAT3 in CD4+ T cells, either left unstimulated or after 20 minutes stimulation with either IL-6 (200ng/ml) or IL21(100ng/ml) in normal controls(n=3). **C.** Representative flow cytometry of flow cytometry analysis of intracellular IL-10 induced expression by CD4 +T cells (CD3+CD4+) in a normal control and patient with ADHIES after stimulating PBMCs with CD2/3/28 with IL 21 (100ng/ml) for 2 days. **D.** Summary % of induced IL-10 production by CD4+T cells after 2 days stimulation of the PBMCs with CD2/3/28 and IL-21(100ng/ml), in normal controls (n= 6) and AD-HIES (n=2). Mann-Whitney: $P<0.05$.

5.4 STAT3-dependent IL-10 induction is best seen in memory T cells (CD45RA negative)

As demonstrated here and elsewhere, naïve CD4+ T cells produce very little IL-10(Figure 5.1 A) (Yssel et al. 1992). We analysed CD4+ T cells according to expression of CD45RA. Memory CD4+ T cells (CD45RA-) responded to STAT3 stimulation with greater IL-10 production than their naïve counterparts. By contrast, IL-10 induction was impaired in memory CD4+ T cells from ADHIES. Thus, STAT3 is required for the augmented component of IL-10 production which is best seen in memory CD4+ T cells. It remains possible that the diminished responsiveness of naïve cells reflects differences in cytokine receptor expression; nevertheless, induction of Il-10 appears to be STAT3-dependent (Figure 5.3 A-C).

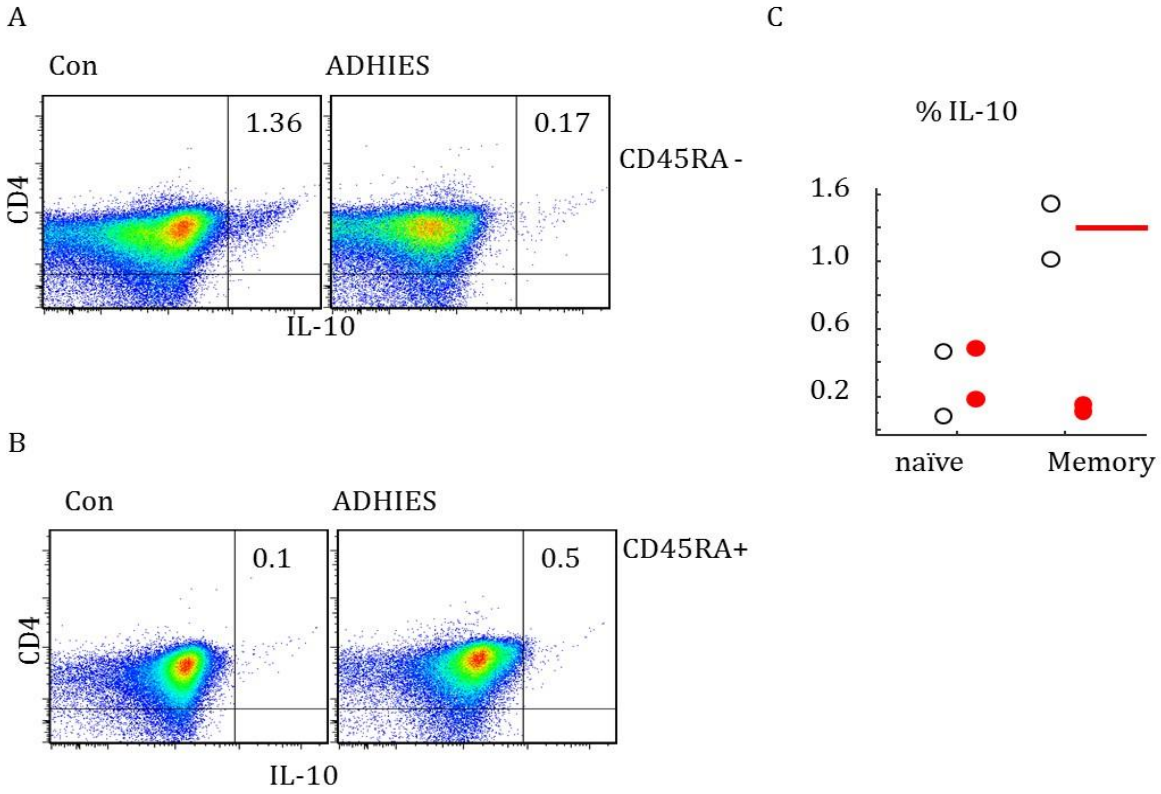

Figure 5-3 Memory human CD4+ T cells can induce IL-10 production in a STAT3 dependent way

A. Representative dot plot of IL-10 production in FACS sorted memory CD4+ T cells (CD4 +CD45RA-) after 2 days stimulation with CD2/3/28 and IL-21 (100ng/ml) in a normal control (con) and a patient with AD-HIES. **B.** Representative dot plot of IL-10 production in FACS sorted naive CD4+T cells (CD4 +CD45RA+) after 2 days stimulation with CD2/3/28 and IL-21 (100ng/ml) in a normal control and a patient with AD-HIES. **C.** Summary % of IL-10 production by FACS sorted CD4+ T cells (naïve vs. memory) after 2 days stimulation with CD2/3/28 and IL-21 in normal controls(black unfilled circles= 2) and AD-HIES (red filled circles, n=2). Mann-Whitney: $P<0.05$.

5.5 IL-10 is not readily expressed by recently induced Th1 or Th2 cells

As noted above, murine studies had identified that IL-10 can be detected in cells cultured under either Th1 or Th2 inducing conditions. We purified naïve CD4+ T

cells and stimulated them with different conditions to induce either Th1 or Th2 cells, and then analysed them for expression of IL-10. We found that the frequency of Il-10+ cells is extremely low in recently induced Th1 and Th2. Moreover, we found no difference in the frequency of these rare IL-10+ cells in Th1 or Th2 cells (Figure 5.4 A-B).

In mice, STAT3-dependent cytokines were shown to be good inducers of IL-10 production by Th2 cells (Altin, Goodnow, and Cook 2012). We therefore investigated this further in human cells. We induced Th0, Th1 and Th2 differentiation from FACS sorted naïve T cells *in vitro* . Cells cultured under Th2 conditions became uniformly GATA3 positive compared to Th1 and Th0 (Figure 5.4 C). Cells generated under each of the three culture conditions were then stimulated with IL-21. We observed sustained STAT3 phosphorylation in each effector cell type in response to this stimulus compared to cells out of culture with no additional IL-21 (Figure 5.5 A).

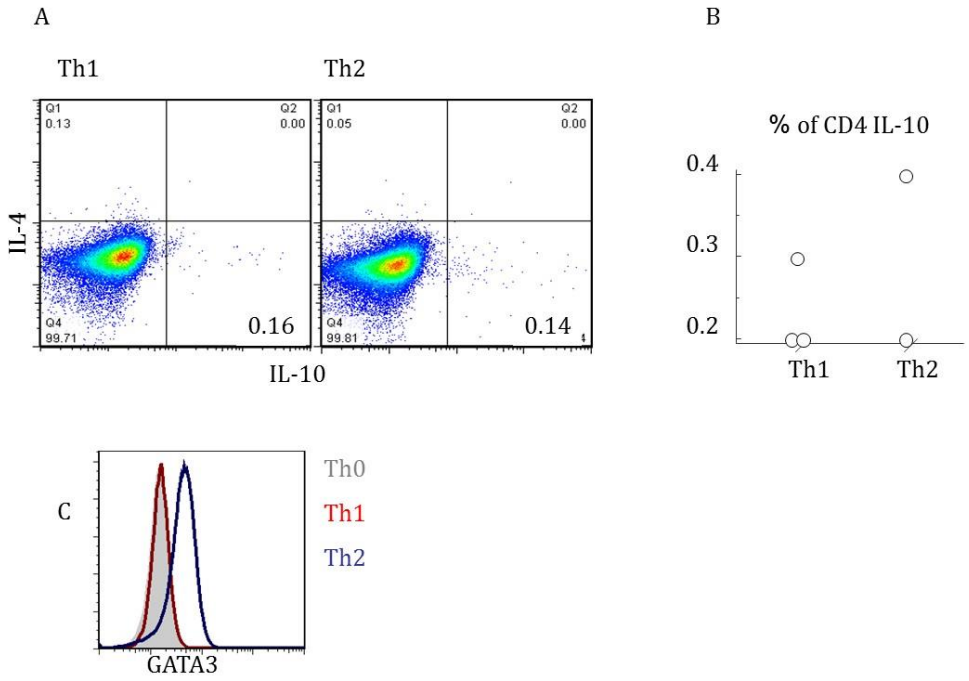

Figure 5-4 IL-10 is not readily produced by recently induced Th1 or Th2

A. Representative dot plot of IL-10 production in FACS sorted naive CD4+ T cells (CD4 +CD45Ro-) after 4 days stimulation in either Th1(CD2/3/28 + IL-12(20ng/ml)) or Th2 (CD2/3/28 +IL-4(20 µg /ml) conditions. **B.** Summary % of IL-10 production by induced Th1 (n=3) versus Th2 in normal controls (n=2). **C.** Histogram of GATA-3 level in induced Th2 versus those Th1 or Th0 (CD2/3/28+IL-2(80ng/ml)).

5.6 IL-10 can be induced in Th2 after further re stimulation in a STAT3 dependent way

Cells cultured under each of the polarizing conditions exhibited sustained pSTAT3 response when incubated with IL-21 for 2 days (Figure 5.5 A), which suggests that STAT3 is active in each subset. Previous reports have shown that activation of either STAT4 or STAT3 in Th1 deviated T cells has the ability to induce IL-10 production. Our data does not support these findings (Figure 5.5 B) and (Figure 5.6A). This might be due to difference in the duration of Th1 induction or the strength of the stimulus as it has been shown that strong STAT4 stimulations needed for IL-10 production by TH1.

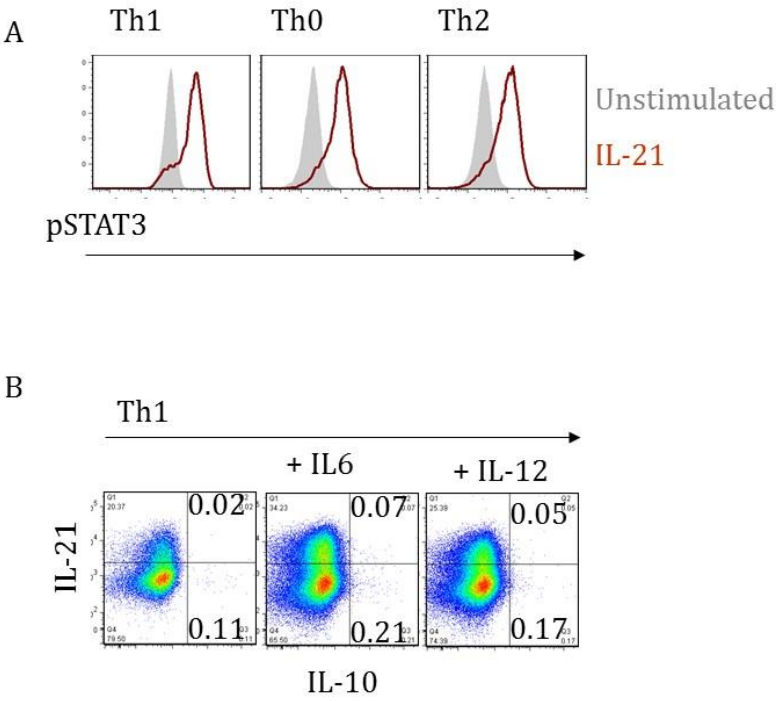

Figure 5-5 IL-10 is not readily induced inTh1 even after activation of STAT3 or STAT4

A. Histogram of p-STAT3 level in newly induced Th1, Th0 and Th2 before and after further re-culture with IL-21(100ng/ml) for 2 days. **B.** Representative dot plot of IL-10 production in FACS sorted naive CD4+ T cells (CD4 +CD45Ro-) after 4 days stimulation in either Th1(CD2/3/28 + IL-12) alone or supplemented with 2 days of IL-6 (200ng/ml) or IL-12 (20 ng/ml).

Next, we investigated the outcome of STAT3 signalling in other effector subsets. First, recently induced Th2, Th1 and Th0 cells were further cultured with IL-2, IL-6 or IL-21 for two days, then harvested and examined for IL-10 production. In comparison with Th1 cells, Th2 cells (Figure 5.6A-C) exhibited greater augmentation of IL-10 production in response to these cytokines. IL-2, IL-6 and IL-21 signal through STAT3, but not exclusively, and therefore, in order to prove that STAT3 is required for the observed response; we have made use of cells with impaired STAT3 signalling from patients with AD-HIES. These cells showed a reduced response compared to normal controls (Figure 5.7A-C).

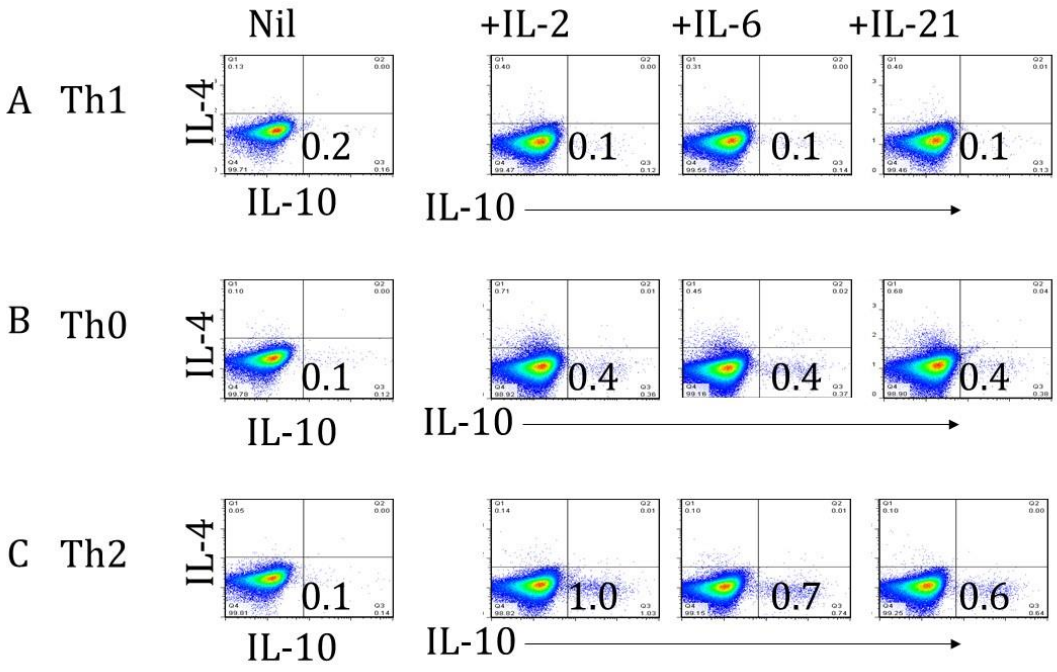

Figure 5-6 IL-10 can be induced in Th2 subsets after STAT3 re-stimulation
Representative dot plot of IL-10 production in FACS sorted naive CD4+ T cells (CD4 +CD45Ro-) after 4 days stimulation in either **A.** Th1(CD2/3/28 + IL-12, 20 ng/ml) or

B. Th0 (CD2/3/28 + IL-2, 80ng/ml) or in **C.** Th2(CD2/3/28 + IL-4(20 µg /ml). All either before or after 2 days stimulation of these subsets with IL-2 (80ng/ml), IL-6 (200ng/ml) or IL-21(100ng/ml) in a normal control.

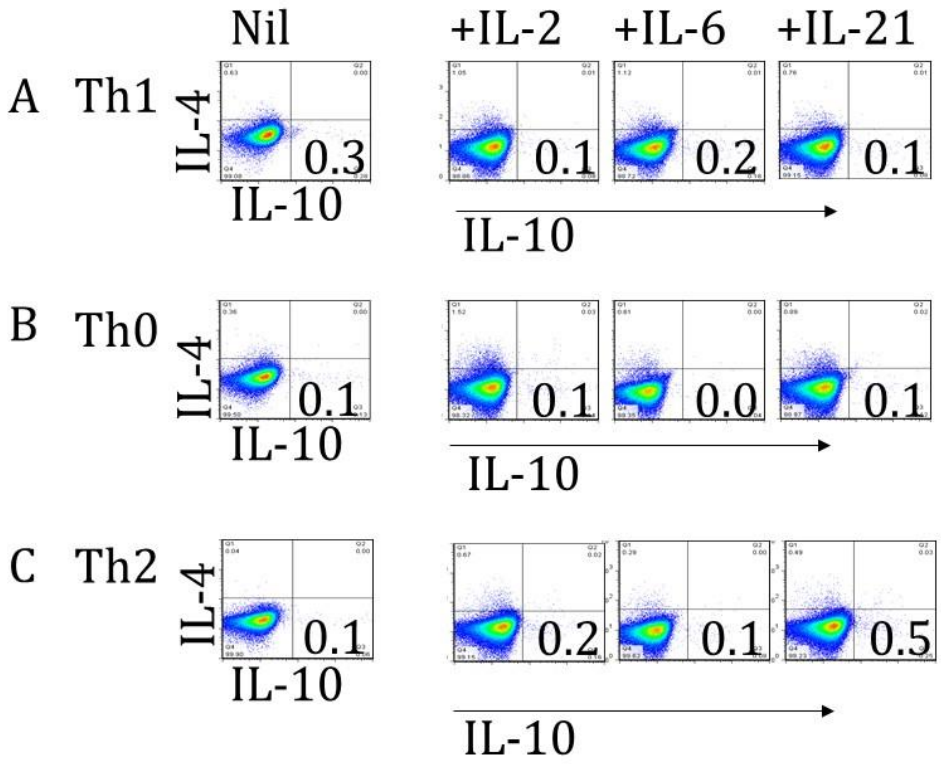

Figure 5-7 IL-10 can be induced in Th2 subsets in a STAT3 dependent way
Representative dot plot of IL-10 production in FACS sorted naive CD4+ T cells (CD4 +CD45Ro-) after 4 days stimulation in stimulation in either **A.** Th1(CD2/3/28 + IL-12, 20 ng/ml) or **B.** Th0 (CD2/3/28 + IL-2, 80ng/ml) or in **C.** Th2(CD2/3/28 + IL-4, 20 µg /ml). All either before or after 2 days stimulation of these subsets with IL-2 (80ng/ml), IL-6 (200ng/ml) or IL-21(100ng/ml) in a patient with AD-HIES.

5.7 Discussion and Summary

We demonstrated here that there is a basal IL-10 production by CD4+ T cells that is STAT3 independent and an inducible component of the IL-10 by Th2 cells, which is STAT3-dependent.

Pathological actions of STAT4, MTOR and STAT3 in human T cell differentiation

AD-HIES patients exhibit some features of chronic Th2-mediated immunopathology, such as rashes, high eosinophil count and very high IgE level. It has been already shown that defective STAT3 signalling in these patients compromises the formation of telorogenic antigenic presenting cells in response to IL-10 (Saito et al. 2011) leading to formation and production inflammatory cytokines.

IL-10 is widely known as an immunomodulatory cytokine that counteracts excessive immunopathology associated with uncontrolled Th1 (Gazzinelli et al. 1996; Li, Corraliza, and Langhorne 1999) (Kuhn et al. 1993; Berg et al. 1996; Sato et al. 2006; Engelhardt et al. 2013; Engelhardt and Grimbacher 2014) (Glocker et al. 2010; Kotlarz et al. 2012; Glocker et al. 2009; Lee et al. 2014) or Th2 induction (Altin, Goodnow, and Cook 2012; Ankathatti Munegowda et al. 2012) (Delprete et al. 1993; Altin, Goodnow, and Cook 2012). IL-10 acts indirectly through antigen presenting cells as well as directly by inhibiting proliferation and cytokine production by T cells.

Moreover, IL-10 production by murine Th1 requires high concentrations of IL-12 and strong TCR stimulation, and depends on STAT4 and ERK signalling (Saraiva et al. 2009). Therefore, it would be expected that individuals with high STAT4 signalling would have higher frequency of IL-10 CD4+T cells. However, the ex-vivo frequency of IL-10 producing cells in the proband and sibling carrying $\boldsymbol{STAT4^{P450S}}$ gain of function was not high. Furthermore, stimulating naïve CD4+T cells under Th1 inducing condition did not yield higher frequency of IL-10 compared to those of Th2, both of which conditions had almost negligible amount of basal IL-10 production. Therefore, in human STAT4 does not appear to support IL-10 production.

The high level of IgE and other manifestation of chronic Th2 immunopathology have not been explained. In animal model it has been shown that chronic Th2 stimulation is associated with induction of IL-10 regulatory Th2 subset. This IL-10 producing Th2 subset despite the fact that they remain Foxp3 negative, they exert regulatory function to counteract the excessive Th2 phenotype.

The data presented here have demonstrated that the STAT3 stimulation in human as well support the formation of this inducible subset from Th2 polarised cell. Therefore, it plausible that, deficiency in this inducible Treg phenotype in Th2 CD4+ T cells is one of the reasons toward the chronic Th1 immunopathology seen in the AD-HIES.

CHAPTER 6 : STAT3 REGULATE CYTOTOXICITY IN CD57+ CD4+ T CELLS

6.1 Introduction

Follicular helper CD4+ T cells (TFH) are specialized T cells accumulate in the follicles of secondary lymphoid organs by virtue of CXCR5 expression and CCR7 down regulation. TFH also express high level of PD-1 (Ansel et al., 1999; Förster et al., 1996; Hardtke et al., 2005) (Breitfeld et al., 2000; C. H. Kim et al., 2001; Schaerli et al., 2000). In humans, but not mice, a significant subset of human TFH cells express CD57 (Bentebibel et al., 2011; C. H. Kim et al., 2001). CD57 expression (encoded by *B3GAT1*) is sometimes considered a hallmark of senescence (Brenchley et al., 2003) and germinal centre CD57+ CD4+ T cells exhibit limited capacity to make IL-2, another characteristic of observed in replicative senescence (Velardi et al., 1986).There is mixed evidence with regard to the relative propensity of the CD57+ and CD57- subsets to provide help to B cells in the tonsil (J. R. Kim et al., 2005; Rasheed et al., 2006), and to date there is no other evidence that this subset is functionally distinct from CD57- TFH cells. Circulating counterpart of TFH cells (cTFH) CD4+ T cells in the blood are also though to am lower level express CXCR5+, PD-1+ and some of this subset are also CD57 +. They are expanded in various autoimmune diseases, including SLE, juvenile dermatomyositis and rheumatoid arthritis (Craft, 2012; Morita et al., 2011; Simpson et al., 2010).

Although originally CD57 was restricted to identify NK cells, now it is well established that it is expressed by CD8+ T cell as well and to a lesser extent by CD4+ T cells. CD57+ CD4+T cell subset is expanded in the context of chronic immune activation(Fleischer 1984) such as in infections with EBV or HIV (Palmer et al. 2005) , some autoimmune diseases such as rheumatoid arthritis (Maeda et al. 2002; Appay 2004) and IgG4 related disease (Mattoo et al. 2016). IL-2-dependent expression of CD57 is observed on a subset of NK cells, which are characterised by attenuated responsiveness to cytokines (Lopez-Vergès et al., 2010). Within the CD8+ T cell and NK cell compartments, CD57 expression also correlates with cytotoxic potential (Chattopadhyay et al., 2009; Lopez-Vergès et al., 2010). In addition, it was shown that CD4 + T cell capable of being cytotoxic and this ability is under the control of CRTAM (Takeuchi et al. 2016).

AD-HIES due to heterozygous missense mutations in *STAT3* results in a cell intrinsic reduction in memory CD4+ T cells, a reduction in CXCR5+ cells as a proportion of CD4+ T cells, (Freeman and Holland, 2010; Holland et al., 2007; Ma et al., 2012; Mazerolles et al., 2013; Minegishi et al., 2007; Siegel et al., 2011) (de Beaucoudrey et al., 2008; Ma et al., 2008; Milner et al., 2008). On the other hand a new syndrome of immunodeficiency as well as autoimmunity and lymphoproliferation has been described in association with *STAT3* gain of function (Milner et al. 2015).

The relationship of dissection of senescence versus exhaustion is not clear for CD4+ T cell and if there is any role for STAT3 in regulating this process is not clear. For this aspect, we revisited the questions of CD57 being a surrogate marker for TFH, possible cytotoxic function of CD57 CD4+ T cells in human by examining both TFH cells from secondary lymphoid tissue (tonsil) and comparing it to circulating TFH (cTFH). Moreover, we were also interested to find out the role of STAT3 in their generation and function of these circulating and cytotoxic CD57+ CD4+ T cells. Therefore, we made use of the LoF and GoF *STAT3* to clarify this.

6.2 CD57 expression correlates with PD-1 in tonsil but not blood

We examined the distribution of follicular T cells in tonsils. Normally, CD57+ CD4+ T cells represent a small proportion of GC TFH cells enriched in the light zone of GCs (Figure 6.1A). By direct immunofluorescence, CD57 expression coincides with PD-1 expression (Figure 6.1B). Concordance of CD57 and high levels of PD-1 expression was confirmed by flow cytometric analysis of single cell suspensions. In fact, co-staining of these two markers has revealed four populations among CD4+ T cells in the tonsil and almost all CD57+ cells express high levels of PD-1 (Figure 6.1C). PD-1^{high} CD57+ CD4+ T cells were mainly present in the secondary lymphoid organs compared to peripheral blood when examining paired tonsils and blood samples from the same donor (Figure. 6.1D). Moreover, expression of CD57 is biased towards CXCR5+ T cells (TFH) in the tonsil compared to blood. In fact in the blood, most CXCR5+ T cells (cTFH) are PD-1^{low}, but CD57 expression identifies cells expressing higher levels of PD-1(Figure 6.1E). After gating on PD-1^{hi} cells in tonsils, approximately 90% of CD4+ T cells co-express CXCR5 irrespective of whether they are CD57+ or CD57- (Figure 6.1F-G). Moreover, the level of CXCR5 expression is similar on PD-1^{hi} cells irrespective of CD57 expression(Haynes et al., 2007) (Crotty, 2011; Ma et al., 2009). CD57 and PD-1 are not expressed by the naïve CD4+ T cells (CD45RA+ compartment) (Figure 6.1F).

We have shown that tonsil CD4+ T cells are classified according to PD-1 and CD57 expression into four distinct memory populations. CD57 identify PD-1 high CXCR5 high TFH cells in the tonsil. In comparison, in the blood, CD57 identifies those rare subsets with the highest PD-1 level and CXCR5+ T cells (cTFH) but it is also expressed in the CXCR5- T cells memory population.

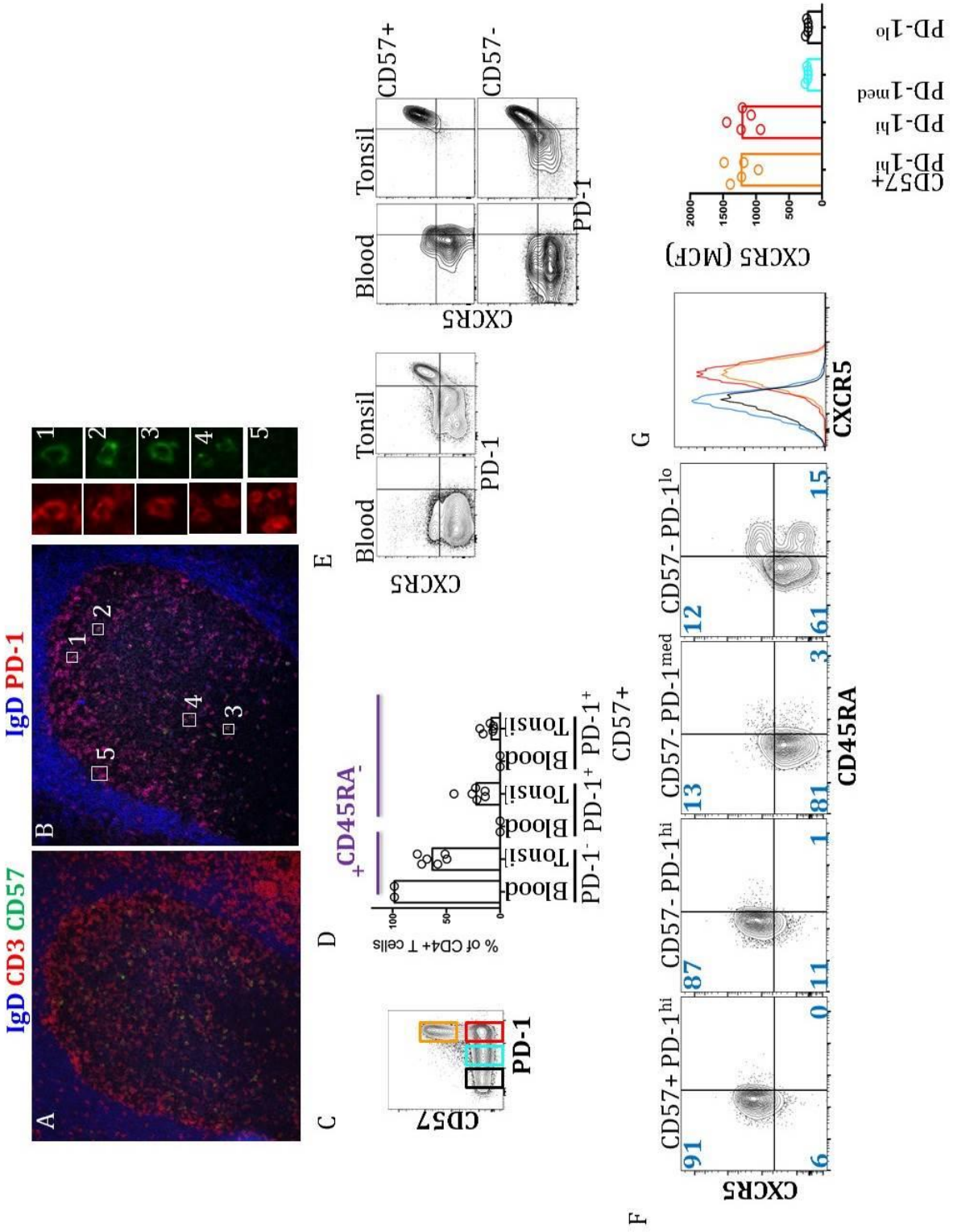

Figure 6-1 Distribution of CD57+ CD4+ T cells in blood and tonsil

A-B. Immunohistological and confocal analysis of CD57+ T cells in sections of tonsil, stained for CD57, CD3 and IgD (**A**) and IgD, PD-1 and CD57 (**B**). High power analysis of individual cells is shown at the left. **C**. Representative flow cytometry analysis of T cell subsets of four CD4+ T cell tonsil subsets determined by PD-1 and CD57 expression in tonsil**. D.** Summary of flow cytometric analysis of single cell suspensions from PBMCs (n=2) and from tonsil (n=6), indicating the proportion of cells within the indicated subsets. **E.** Analysis of CXCR5 and PD-1 expression in a paired sample gated on total CD4+ T cells and then on CD57 status. **F.** Analysis of CXCR5 and CD45RA expression by the four tonsil subsets obtained from the co-staining of PD-1 and CD57. **G.** Overlay histogram of the level of CXCR5 expression by the four subsets as defined in C. Summary of mean fluorescence level of CXCR5 expression in the four defined subset as in C.

6.3 CD57+ CD4+ T cells exhibit attenuated cytokine production

Now, having shown that CD57 identifies TFH cells (PD-1^{hi} cells and CXCR5+ T cells) in the tonsil but identify cells with PD-1^{hi} among cTFH and non cTFH, we were interested to examine the functional ability of these different subsets (according to PD-1 and CD57) by examining the cytokine expression. Initially examining paired blood and tonsillar samples from the same donor, has revealed a significant bias towards IL-17, IL10, and IL-21 production in tonsil (Figure 6.2A). In other words, there is a relative increase in production of STAT3-dependent cytokines within tonsil. We also examined cytokine expression in different four tonsil subsets according to CD57 and PD-1 expression. PD-1^{hi} are relatively enriched for IL-4 (Figure 6.2B-C). Interestingly, while IL-4 predominates in CD57+ cells relative to other cytokines, IL-10 and IL-21 predominate in the CD57- PD-1^{hi} subset (Figure 6.2B-C). Although PD-1^{hi} CD4+ T cells are rare in peripheral blood, a relatively high proportion of them produce IL-21 and IL-10 (Figure 6.2D). Indeed, expressed as a proportion of the total subset, the bias towards IL-21 production by PD-1^{high} cells is even greater in blood than tonsil. Interestingly, cytokine production is attenuated in the CD57+ subset. Relative to PD-1^{hi}

CD57- cells, far fewer tonsil cells in the CD57+ PD-1^{hi} double positive fraction express either IL-10 or IL-21.

TFH populations can also be sub classified according to chemokine receptor expression, which is sometimes taken as a surrogate for cytokine production and capacity to provide help to B cells. Consistent with the intracellular cytokine analysis, few CD57+ cells express CXCR3, and almost none express CCR6 (Figure 6.2E). Expression of these chemokine receptors is more abundant on tonsil CD4+ T cells with lower levels of PD-1 expression (Figure 6.2E-F). Overall, PD-1^{hi} cells exhibit a similar cytokine phenotype in blood and tonsil, and CD57 identifies a subset with attenuated cytokine production and chemokine receptor expression.

Pathological actions of STAT4, MTOR and STAT3 in human T cell differentiation

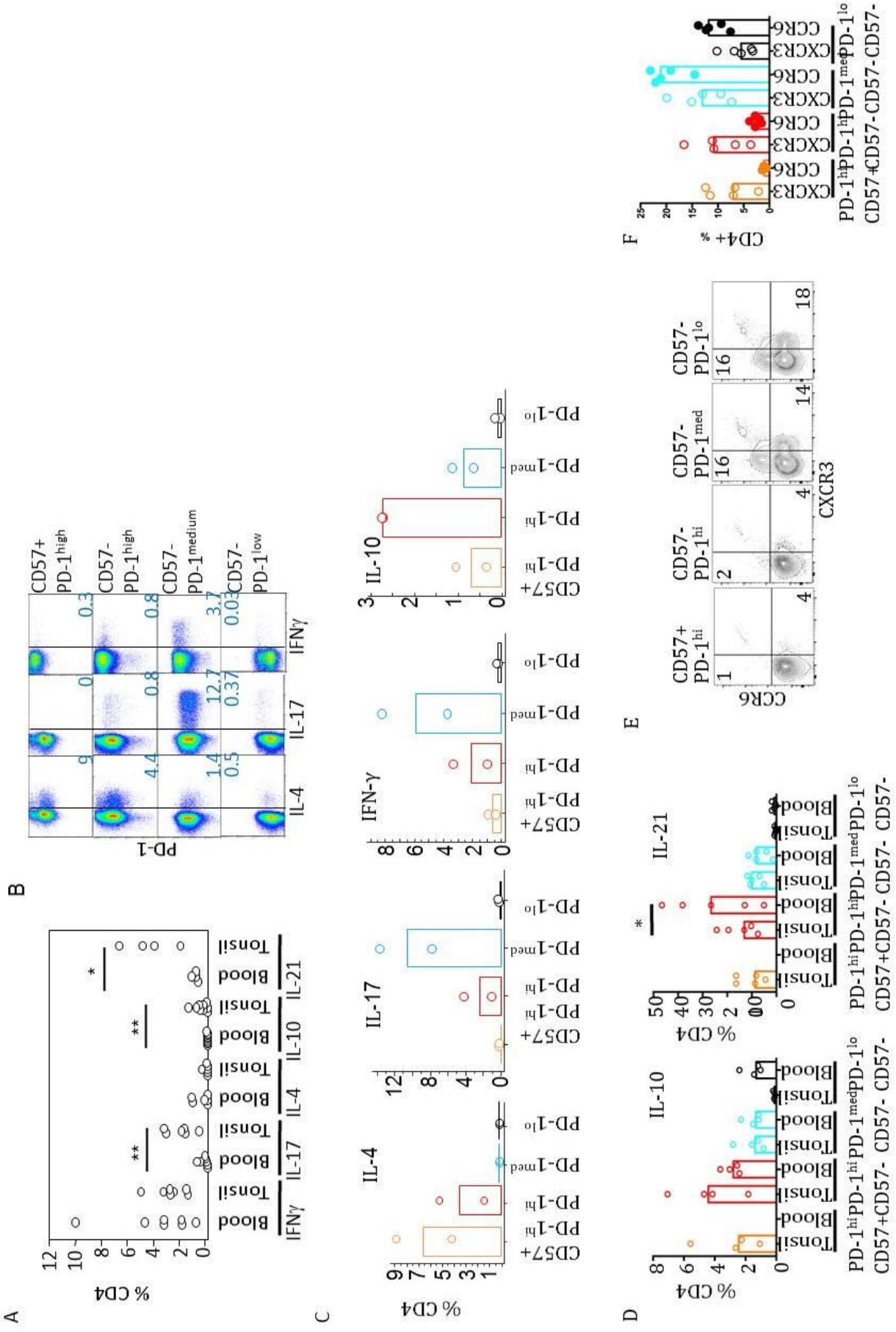

Figure 6-2 Cytokine production by cell according to PD-1 and CD57 expression
A. Summary plot of the relative abundance of intracellular cytokine as indicated in paired blood and tonsils (n=7 for IFN-γ, IL-17, IL-4, IL-10 and n=4 for IL-21 assessment). **B.** Representative plot of intracellular cytokine detection in FACS sorted four tonsil subsets according to the CD57 and PD-1 expression as in (figure 5.1 C). **C.** Summary plot % of CD4 + T cells positive for indicated intracellular cytokine in the four subsets in the four FACS sorted tonsil subsets (n=2) as in (figure 5.1 C) .**D.** Summary plot of the relative abundance of intracellular IL-10 (n=4) and IL-21 (n=5) production by the four subsets according to PD-1 and CD57 expression as in (figure 5.1 C) in paired blood and tonsil. Each symbol represents the result from an individual. **E.** Representative flow cytometry analysis of expression of CXCR3 and CCR6 in different subsets of CD4+ T cells defined in(figure 6.1 C) and the summary data for similar analysis (F), n=5.

6.4 STAT3 responsiveness is impaired in PD-1high CD4+ T cells

Having established that STAT3-dependent cytokines are enriched within tonsil, and that two of these (IL-10 and IL-21) are abundantly expressed within PD-1^{hi} CD4+ T cells in blood and tonsil, we proceeded to examine responsiveness to STAT3-dependent cytokine stimuli within the CD4+ T cells compartment. We sorted tonsil CD4+ T cells into four populations according to PD-1 and CD57 expression, and examined STAT3 phosphorylation, expression of IL-4 and IL-10, and induction of IL-10 in response to IL-6 or IL-21. We observed an inverse relation between PD-1 expression and pSTAT3 induction in response to IL-21, with virtually no expression in PD-1^{high} CD57+ cells containing pSTAT3 (Figure 6.3 A).The pSTAT3 response to various cytokines within Tregs is shown for comparison. We observed a reduction in pSTAT1 signalling in response to IFN-γ in PD-1^{hi} cells, suggesting that this state of unresponsiveness is not restricted to STAT3 signalling cytokines. STAT3 signalling is not globally compromised, however, as pSTAT3 was detected after stimulation with IL-6.

Next we examined the same populations of cells for expression of IL-4 and IL-10 analysed immediately ex vivo, or after stimulation *in vitro* (Figure 6.3 B). As noted earlier, IL-10 production as a proportion of CD4+ T cells was greatest in PD-1^{hi} CD57- T cells, and was attenuated in the CD57+ subset. Activation of FACS sorted CD4+ T subsets with anti-CD2/3/28 and either IL-6 or IL-21 resulted in induction of IL-10 by PD-1^{med} cells. We saw no cytokine induction in CD57+ PD-1^{hi} cells (Figure 6.3 B).

Next, we examined the same purified CD4+ T cell populations for proliferation and cytokine production in response to stimulation with anti-CD2/3/28 in the absence of exogenous cytokines (Fig. 6.3C). CD57- CD4+ T cell population exhibited a similar response to stimulation irrespective of PD-1 expression. By contrast, CD57+ cells exhibited a marked defect in proliferation. Fewer cells entered cycle, and those that did progressed through fewer cell divisions than CD57- CD4+ T cells. Examination of cytokine production confirmed the relative unresponsiveness of CD57+ cells. Interestingly, we observed IL-21 induction in the absence of any polarizing stimulus, and this was more marked in PD-1^{low} cells (Figure 6.3 C).

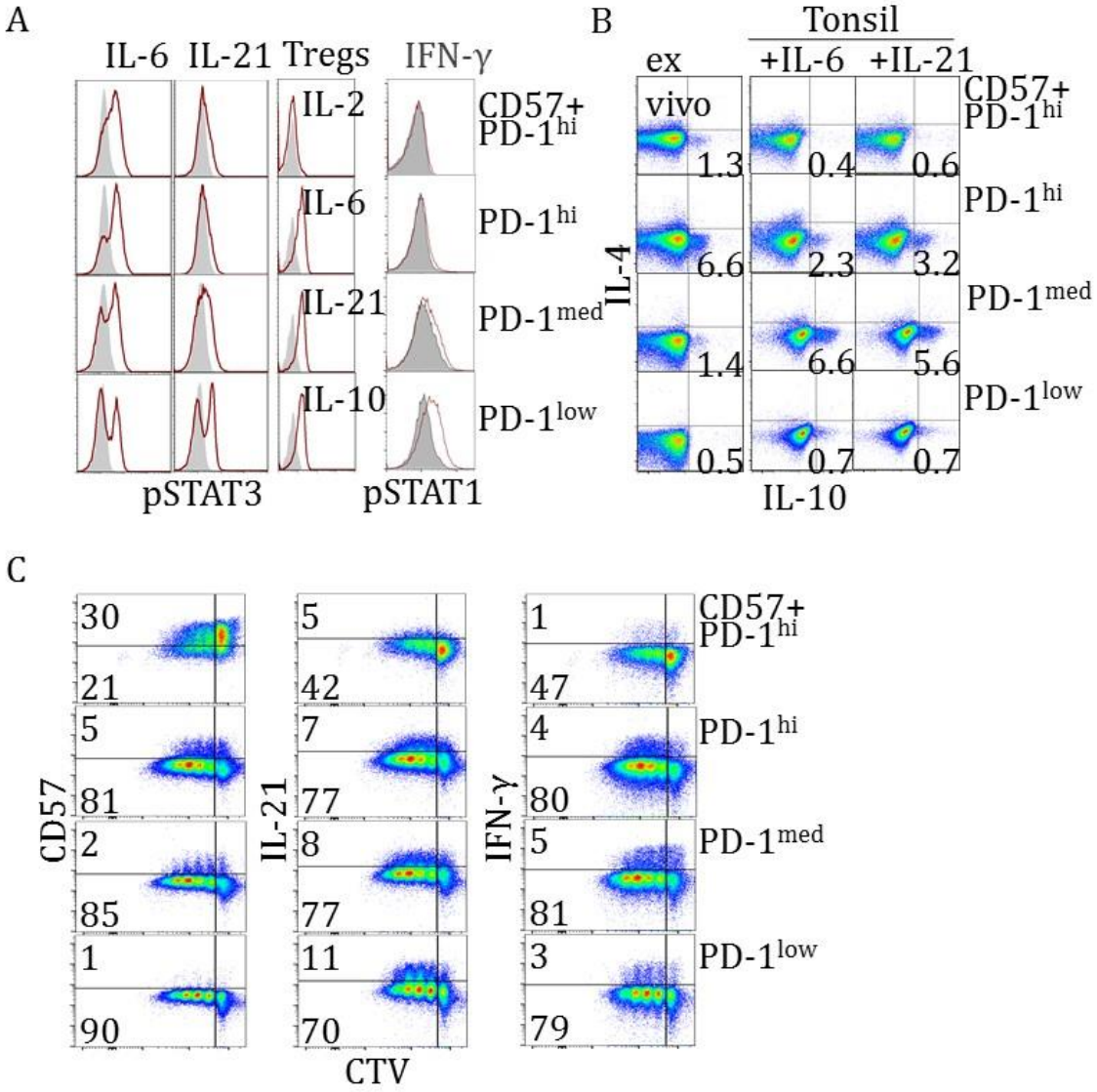

Figure 6-3 Responsiveness of CD57+ CD4+ T cells

A. Overlay histogram of phosphorylation of STAT3 and STAT1 after stimulation of each PD-1 and CD57, CD4+ T cell subset with either IL-6(200ng/ml), IL-21(100ng/ml) or IFN-γ(10 U/ml). Responses in Treg (CD4+CD25+CD127-) are shown for comparison. Unstimulated (filled grey) and stimulated (unfilled red). **B.** Flow cytometry analysis of ex-vivo and induced intracellular IL-10 and IL-4 expression after stimulation of purified subsets with either IL6 (200ng/ml) or IL-21(100ng/ml) for 2

days. **C**. Flow cytometry analysis of proliferation and induction of intracellular expression of IL-21 or IFN-γ by purified cells from each of the indicated subsets.

6.5 CD57+ CD4+ PD-1high T cells are not T follicular regulatory cells

Since CD57+ cells irrespective of PD-1 expression, appear to be less able to make cytokines or proliferate, we wondered whether this subset might harbour T follicular regulatory cells (TFR). This does not appear to be the case, since CD25+ CD127^{lo} cells are predominantly CD57- (Figure. 6.4 A-B). Conversely, CD57+ cells are predominantly located in the CD25- CD127- compartment. In addition, the small population of CD25+ CD127- cells in tonsil those are CD57 +, are FOXP3- in the tonsil and in the blood (Figure. 6.4 C). Gating on TFH by PD-1 and CXCR5, approximately 24% of CD25+ cells express CD57, while 40% of the much larger CD25- fraction are CD57+(Figure. 6.4 D-E). Consistent with data shown above, analysis of conventional Tregs and conventional memory T cells shows that IL-10 production is confined to the CD57- population (Figure. 6.4 E-G).

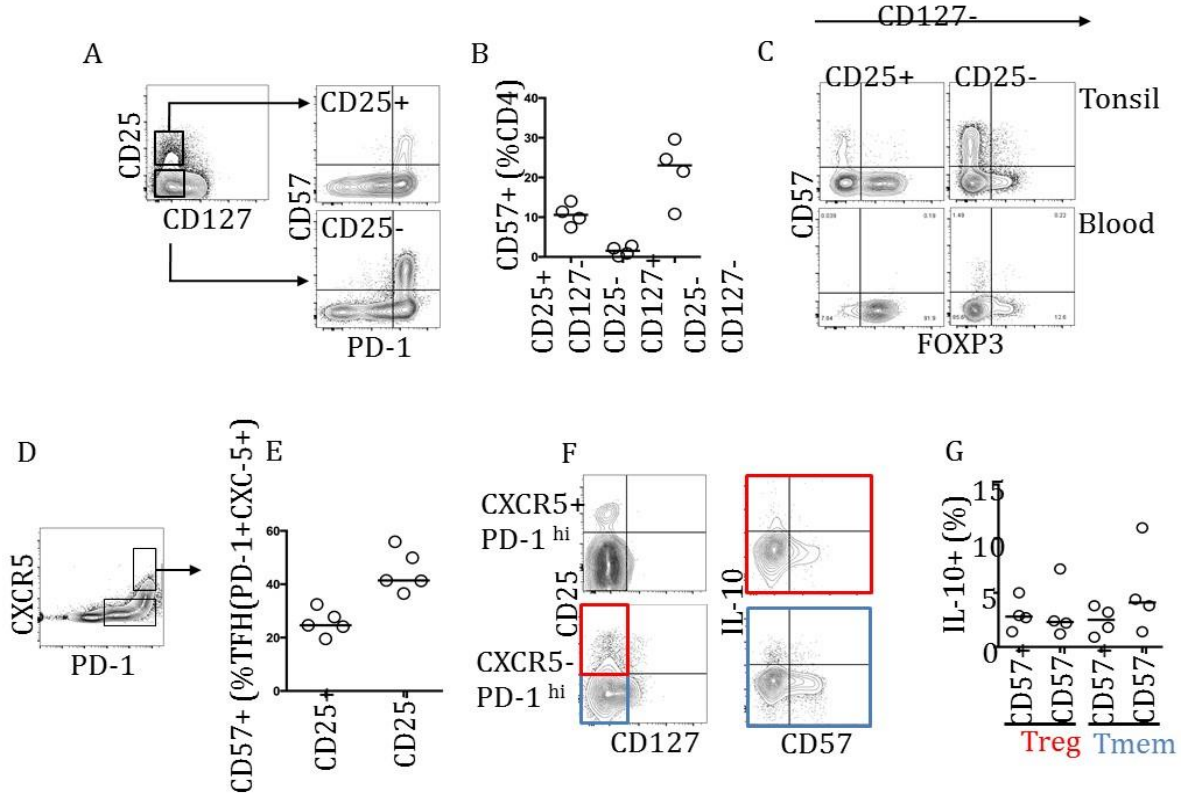

Figure 6-4 CD57+ CD4+ T cells are not enriched with TFR cells

A. Flow cytometry analysis of CD4+ tonsillar T cells that are either CD25+ CD127- or CD25- CD127- cells for expression of CD57 and PD-1. **B.** Summary plot of A (n=4). **C.** Flow cytometry analysis of FOXP3 and CD57 expression in CD127- that are either CD25+ or CD25- cells obtained from either blood or tonsil. **D.** Flow cytometry analysis of TFH cells (gated on CXCR5 and PD-1) for proportion of CD25 and CD57 and **E.** Summary plot proportion of CD57+ in either CD25+ or CD25- cells (n=4)TFH cells (CXCR5+ PD-1^{hi}) in the tonsil. **F.** Analysis by flow cytometry for CD57 and intracellular expression of IL-10 cells after gating on indicated TFR phenotype CXCR5+PD-1^{lo} according to CD25 and CD127 expression**. G.** Summary plot proportion of F (n-4).

6.6 CD4+ CD57 TFH cells exhibit a cytotoxicity gene expression signature

So far we shown that regardless of CD57 status, PD-1^{high} CD4+ T cells have reduced cytokine production and proliferation capacity. Therefore, failing to demonstrate any difference in these two aspects between CD57+ and CD57 – CD4+ T cells. We then investigated possible functional differences between CD4T cells that are either CD57+ or CD57- cells by examining their gene expression signature. We purified PD-1^{high} CD4+ T cells from tonsils according to expression of CD57. For comparison, we purified PD-1^{lo} cells (CD45RA-) from the same tonsils, and analysed them for gene expression using RNASeq. Overall, we observed considerable similarity between expression patterns in CD57+ and CD57- PD-1^{hi} CD4+ T cells, nevertheless, we identified some important differences (Figure 6.5 A) Consistent with cellular phenotyping, we observed a relative bias towards IL-17 and IFN-γ within CD57- cells. By contrast, we noted increased expression of *EOMES*, *WNK2 and PRDM8* in the CD57+ subset (Figure 6.5 B). Analysis of gene expression in each PD-1^{hi} population relative to PD-1^{lo} cells identified a number of additional possible signals. Genes exhibiting greater expression in CD57+ cells included *GZMA*, *CRTAM*, and *IL21*, while

LAG3, TNFRSF11A, CD248 were expressed at lower levels in the CD57+ subset (Figure 6.5 C).

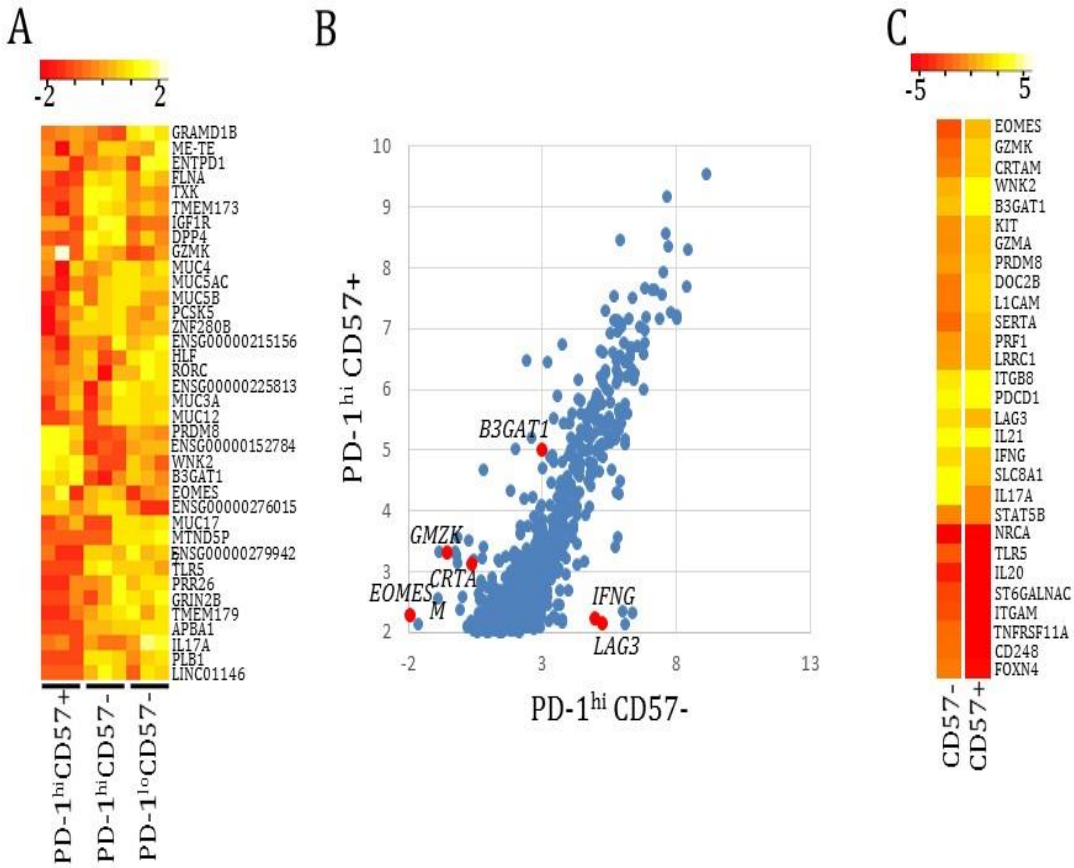

Figure 6-5 Analysis of global gene expression

PD-1hi CD57+, PD-1hi CD57- and PD-1lo CD57- cells were purified and then analysed for gene expression by RNASeq. **A.** Comparison of each subset. Results show three biological replicates in each group. **B.** Analysis of relative expression in PD-1hi CD57+, PD-1hi CD57- cells. Genes of interest are highlighted (red).**C.** Comparison of PD-1hi CD57+ with PD-1lo and PD-1hi CD57- with PD-1 lo reference data.

CRTAM (MHC class-I restricted T cell associated molecule) has been reported recently as a master regulator of CD4+ cytotoxic T cells in mice (Takeuchi et al., 2016). We

proceeded to analyse CD57+ and CD57- cells a for intracellular CRTAM protein expression. In tonsil, only a small proportion of CD57+ cells co-express CRTAM, but very few CRTAM+ cells are present in the CD57- population (Figure 6.6 A). Thus, the proportion of CRTAM+ cells in the CD57+ population is about twice that in CD57- cells in the tonsil (Figure 6.6 B). Similar analysis of circulating CD57+ cells revealed a more substantial bias to CRTAM expression (Figure 6.6 A-B). As noted above, the CD57+ population in blood is small, nevertheless, approximately 30% of these cells express CRTAM protein. As a result, the proportion of CD57+ cells that are CRTAM+ cells is approximately 8-fold greater than in CD57- cells.

CRTAM is identified as a master regulator of cytotoxic CD4+ T cells, we also examined CD57+ and CD57- cells for additional cytotoxicity related proteins. Consistent with gene expression, we observed a small proportion of granzyme-A cells within the CD57+ compartment in tonsil, and a more substantial proportion on circulating CD57+ cells (Figure 6.6 C-D). On analysis of all four tonsil subsets (defined by PD-1 and CD57-), we observed most granzyme positive cells are located within the CD57+ PD-1^{hi} population (Figure 6.6 D). Similarly, in blood, almost all granzyme-A cells are CD57+.

We tested whether expression of CRTAM and granzyme, together with the cytotoxicity transcriptional signature, translates into a granzyme-perforin mediated functional cytotoxicity phenotype. We purified CD57+ and CD57- PD-1^{hi} CD4+ T cells and co-cultured these with B cell targets. After 1 hour, pre-labelled target cells were analysed for intracellular perforin. We observed a small but consistent increase in cytotoxicity mediated by CD57+ cells (Figure 6.6 E).

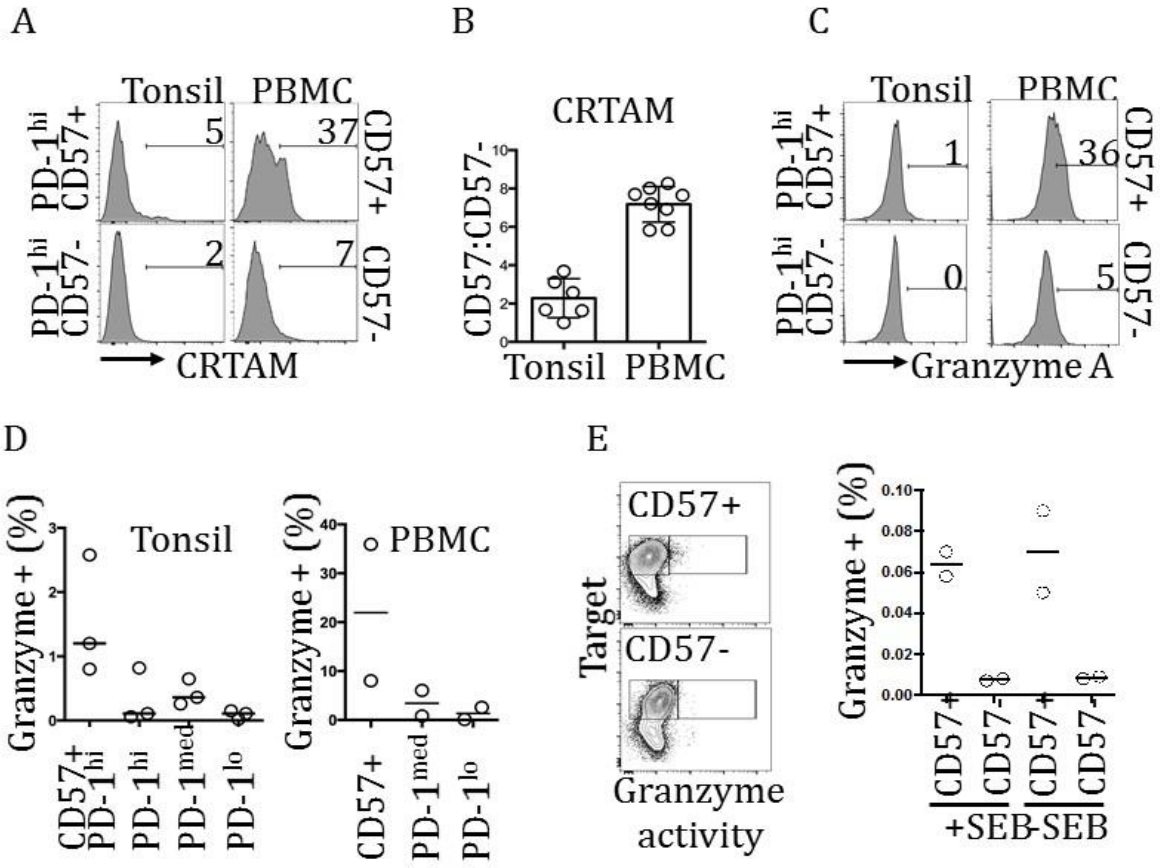

Figure 6-6 Cytotoxicity phenotype in CD57+ CD4+ T cells

A. Flow cytometry analysis of intracellular CRTAM expression in tonsil and blood CD4+ T cells. Tonsils gated on PD-1hi CD57- or PD-1hi CD57+; PBMCs gated on CD57+ or CD57-. **B.** Summary plot of relative CRTAM expression in each subset gated in (A) in blood (n=8) and tonsil (n=6). **C.** Flow cytometry analysis of intracellular granzyme-A expression in tonsil and blood. Tonsils gated on PD-1hi CD57- or PD-1hi CD57+; PBMCs gated on CD57+ or CD57-. **D.** Summary of relative granzyme positive cells in PBMC (n=2) and tonsil (n=3) according to PD-1 CD57 subsets. **E**. Flow cytometric analysis of cytotoxicity of target cells co-cultured with CD57+ or CD57- CD4+ T cells, with summary plot of similar experiments (right panel, n=2).

6.7 STAT3 regulates CD4+ T cell cytotoxicity independently of exhaustion

Finally, we were interested to investigate possible pathways that regulate abundance of CD57+ CD4+ T cells both within the CXCR5+ follicular compartment, and in the periphery. Our previous results had demonstrated that in humans, *STAT3* haploinsufficiency (loss-of-function, LoF), observed in patients with autosomal dominant hyper IgE syndrome, results in a reduction in CXCR5+ CD4+ T cells in blood(Ma et al., 2012) (Figure 6.7 A-B). More comprehensive analysis of blood from these patients reveals that despite the deficiency in cTFH, there is an increase in PD-1^{hi} CD4+ T cells (Figure 6.7A). Indeed, when PD-1 ^{high} cells are expressed as a proportion of each parent population, STAT3 deficiency results in a relative increase of PD-1^{hi} cells in both CXCR5+ and CXCR5- populations in the peripheral blood (Figure 6.7 C-D). The proportion of CD57+ cells is increased in the peripheral blood, and as in normal tonsil, this increase is predominantly within the PD-1^{high} population, but unlike in tonsil, in *STAT3* LoF these cells are mostly both CXCR5+ and CXCR5- populations (Figure 6.7 A).

We also examined blood from rare individuals with *STAT3* gain-of-function (GoF) mutations, which confers a phenotype of antibody deficiency and autoimmunity (Flanagan et al., 2014; Haapaniemi et al., 2015; Milner et al., 2015). Thus, as with *STAT3* LoF, lymphocytes from these patients are expected to be under chronic antigenic stimulation (either self- or foreign antigen). In CD4+ T cells *STAT3* GoF patients, we observed an expansion of cTFH cells (Figure 6.7A-B). Relative to normal blood samples, this is associated with an increase in the proportion of PD-1^{hi} cells, but not as marked as observed with *STAT3* LoF (Figure 6.7C-D). By contrast, there was no expansion of CD57+ cells (Figure 6.7 A). Furthermore, the distribution of CD57 was similar to that observed in normal blood, with similar low CD57 expression in both PD-1^{hi} and PD-1^{lo} CD4+ T cells. The surface phenotype suggested that *STAT3* LoF results CXCR5- cells adopting a phenotype similar to that observed in CXCR5+ cells (in tonsil) compared to normal individuals (Figure 6.7 C-D),more of exhausted phenotype marked by PD-1 ^{high} and CD57+ regardless of the CXCR5 status. Consistent with this

observation, analysis for CRTAM and granzyme expression is similar in tonsil and *STAT3* LoF PBMC. By contrast, *STAT3* GoF results in a substantial increase in CRTAM and granzyme-A expression similar to seen in normal controls (Figure 6.7 F-G). Taken together, these findings reveal that the CD57+ exhaustion phenotype and cytotoxicity phenotypes can be dissociated, and that normal STAT3 promotes cytotoxicity without exhaustion.

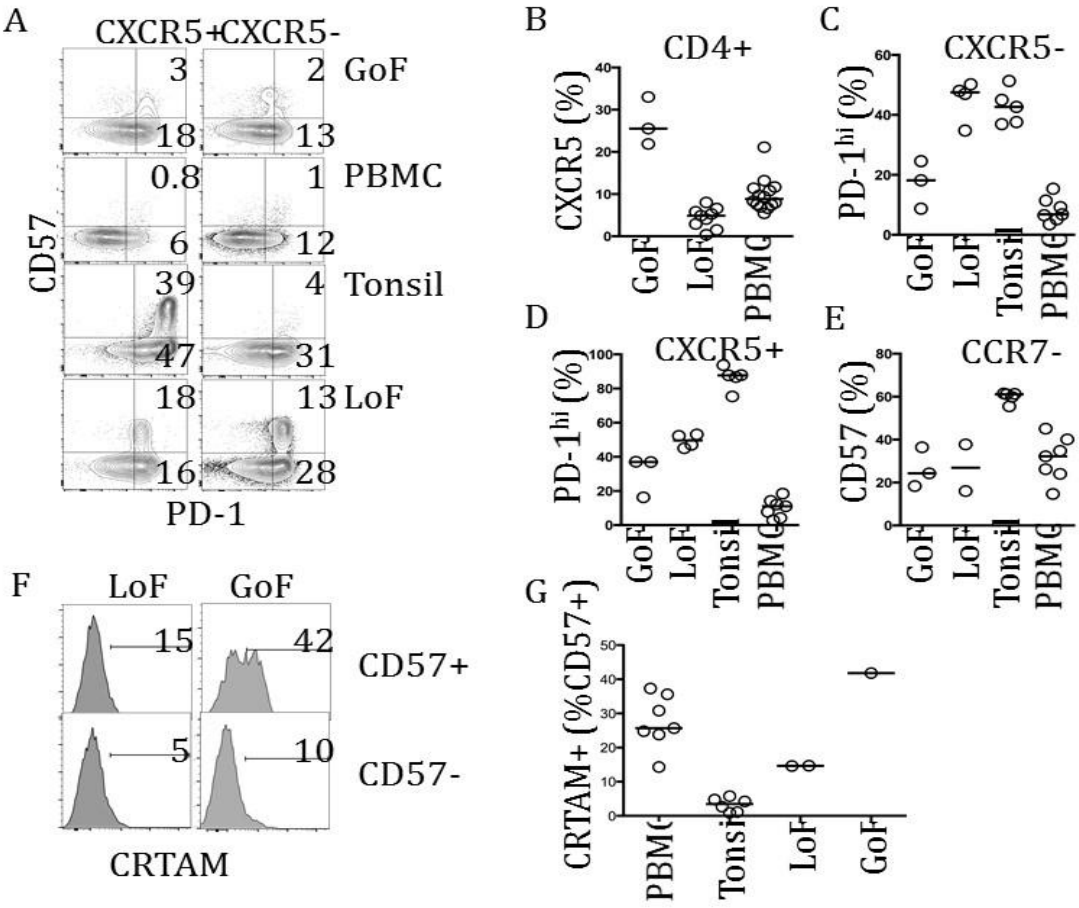

Figure 6-7 STAT3 influence on CD4+ T cell cytotoxicity

A. Flow cytometry analysis of abundance of CD57+ cells in CXCR5+ and CXCR5- compartments in blood from patients with *STAT3* loss-of-function (LoF), gain-of-function (GoF) mutations, or from PBMC of normal donors, or tonsil. **B**. Summary plot of TCH cells (CXCR5+ CD45RA-) cells from *STAT3* GoF(n=3), *STAT3* LoF (n=9)or normal donors. **C-D.** Summary plot of abundance of PD-1hi cells (as a percentage of total CD4+ T cells) in CXCR5- **(C)** and CXCR5+ **(D)** compartments in PBMCs from

STAT3 GoF(n=3), LoF(n=4), healthy donors(n=7) or tonsil(n=5). **E.** Summary plot of abundance of CD57+ cells in CCR7+ compartments in PBMCs from *STAT3* GoF(n=3), LoF(n=2), healthy donors(n=7) or tonsil(n=5). **F-G.** Representative flow cytometry analysis of intracellular CRTAM expression in PBMCs from *STAT3* GoF and *STAT3* LoF patient, with summary plot of similar analyses **(G)** *STAT3* GoF(n=1) and *STAT3* LoF patient(n=2), healthy donors(n=7) or tonsil(n=6).

6.8 Discussion

We have presented evidence that CD57 expression identifies cells with cytotoxic potential, and a subset of these cells demonstrates overt cytotoxic activity. This function is flagged by expression of CRTAM, recently identified in mouse CD4+ T cells, as a master regulator of the CD4+ cytotoxic T cell subset (Takeuchi et al., 2016). Our findings are therefore consistent with CRTAM performing a similar function in human CD4+ T cells. We show that CD57 identifies cells with cytotoxic potential in both blood and tonsil, although this association is accentuated in circulating cells. Our findings indicate that while CD57 identifies the subset of CD4+ T cells that are either have exhausted phenotype or cytotoxic function; this distinction can be dissociated according to the intensity of STAT3 signalling.

Using blood and tonsil samples from the same donors, we show that there is an overall deviation towards increased PD-1 expression in tonsil relative to blood in each well-defined T cell subset analysed. This means there is also a much greater abundance of PD-1 ^{high} cells in tonsil than blood. Furthermore, T cell function varies with PD-1 expression. We show that cytokine production varies qualitatively according to PD-1 expression, although the level of PD-1 expression does not correlate well with proliferative potential. Thus, there is no conclusive evidence that PD-1 by itself reads out exhaustion. Nevertheless, a subset of tonsillar CD4+ PD-1 high T cells express CD57, and these cells acquire an exhausted phenotype of diminished proliferative potential, and reduced chemokine receptor expression, and cytokine production. It has been known for some time that this subset produces less IL-4 than their CD57-

counterparts (Bowen et al., 1991; Butch et al., 1993). CD57+ cells simultaneously adopt the potential for cytotoxicity. In tonsil, where CD57 only occurs on PD-1 high cells, the cytotoxic potential is also attenuated relative to CD57+ counterparts in blood, where PD-1^{hi} cells are rare.

Senescence and exhaustion within the CD4+ T cell compartment remain incompletely understood. There is much more information about these two phenotypes in the CD8+ T cell compartment, and here, exhausted cells express a range of inhibitory molecules, including PD-1, TIM3, CD244, and CTLA4, and generally exhibit a relative defect in IL-2 production(Crawford et al., 2014). In chronic viral infection, exhausted T cells are characterised by increased expression of IL-10, and chronic murine viral infection is associated with enhanced IL-21 production by CD4+ T cells (Elsaesser et al., 2009). Indeed, chronic infection has been reported to promote adoption of the TFH phenotype (Fahey et al., 2011). Since mice do not express CD57, these studies have not determined whether exhaustion or indeed the TFH phenotype that arises in states of chronic antigenic stimulation might be associated with cytotoxicity in a subset of these cells. Within the human NK cell compartment, CD57 expression is associated with lytic activity, but with decreased responsiveness to cytokines (PMID20733159), and is marked by induction of Eomes (Lopez-Vergès et al., 2010).

We demonstrate that there is not only a bias towards more PD-1 expression in tonsil (for any given subset), but also a bias towards production of STAT3-depependent cytokines. It is of interest, and somewhat paradoxical that we also show that the cytotoxic potential of CD57+ CD4+ T cells is enhanced by STAT3 signalling, with increased expression of CRTAM and granzyme-A, whereas impaired STAT3 signalling is associated with increased abundance of CD57+ CD4+ T cells, but not an increase in cytotoxicity. As TFH cells acquire the PD-1 ^{high} CD57+ phenotype they also become refractory to STAT3 signalling. The bias towards STAT3-dependent cytokines within tonsil implies that there are other CD4+ T cell subsets that respond well, and this appears to be the case based on isolation and stimulation of cells according to PD-1 expression. Indeed, STAT3 appears to be important for TFH to form in the first place,

as CXCR5+ cells are less abundant in patient with *STAT3* LoF, and as we show here, more abundant in patients with *STAT3* GoF. Nevertheless, within the TFH population, STAT3 refractoriness occurs, and this is marked by CD57 expression, high level PD-1, and coincides with adoption of potential for cytotoxicity. Consistent with evidence from NK cells and mouse models of chronic viral infection, PD-1 high phenotype is associated with maximal IL-10 and IL-21 production, but once these cells express CD57 they become refractory to signalling, and cytokine production.

Previous investigations have suggested that CD57+ and CD57- cells are similar with regard to B cell help (J. R. Kim et al., 2005; Rasheed et al., 2006). Furthermore, both CD57+ and CD57- cells express comparable levels of BCL-6 (not shown). Consistent with this similarity in B cell help, the difference between CD57+ and CD57- cells, at least by transcription, is very small yet significant, with up regulation of EOMES and CRTAM. CRTAM had previously been identified in CD8+ T cells and NK cells, but a small fraction (2-5%) of mouse CD4+ T cells are CRTAM+, and also express Eomes and granzyme-B. Transgenic expression of CRTAM indicates that it is the master regulator of this CD4+ T cell fate. Although CD57+ cells in blood are not universally PD-1^{hi}, they do exhibit the same cytotoxic phenotype. Indeed, we observed a higher proportion of cells expressing of markers of cytotoxicity, granzyme and CRTAM, in blood. On the other hand, CD57+ CD4+ T cells are much more abundant in tonsil than blood. Thus, in blood there appears to be a constraint on CD57+ cell formation, which is not present in tonsil. Conversely, CD57+ cells in blood appear to adopt the cytotoxic potential more readily than their tonsillar counterparts.

STAT3 appears to be central to regulation of these phenotypes. As noted, STAT3 regulates adoption of the CXCR5+ phenotype(Ma et al., 2012). In addition, evidence presented here indicates that STAT3 promotes adoption of CRTAM and granzyme expression. By contrast, loss of STAT3 signalling, either genetically specified or acquired within follicles, correlates with PD-1 and CD57 expression, but not accentuation of the cytotoxic phenotype. Indeed, while CD57 expression marks cells with a cytotoxic potential, the abundance of CD57+ CD4+ T cells does not predict the

proportion that adopt the cytotoxic phenotype. In both tonsils (TFH cells), and analysis of circulating T cells from patients with *STAT3* LoF , PD-1+ CD57+ cells are abundant, but the CRTAM phenotype is relatively uncommon in these cells. By contrast, *STAT3* GoF appears to promote the cytotoxic phenotype without expansion of the CD57 population in which these cytotoxic T cells arise.

From our analysis we infer that the follicular compartment guards against cytotoxicity, while at the same time, cells adopt a phenotype of exhaustion more readily than their circulating counterparts.

One possible scenario is that the balance between exhaustion with or without cytotoxicity hinges on the relative abundance of IL-2 and STAT3-mediated cytokine signals. IL-2 has been shown to promote cytotoxicity amongst NK cells, that can then adopt a more cytotoxic phenotype, and simultaneously become CD57 positive (Lopez-Vergès et al., 2010). In STAT3 deficiency, IL-2 effects predominate and promotes acquisition of CD57 irrespective of whether they are CXCR5+ or negative. Indeed, in STAT3 deficiency, this CD57 phenotype dominates in both compartments of CXCR5+ and CXCR5- . On the other hand, normal to high STAT3 signalling appears to promote cytotoxic phenotype, independently of CD57 adoption. In *STAT3* GoF, IL-2 signal is not altered.

What has emerged from our analysis is that irrespective of whether CD57 identifies exhausted or senescent T cells, their cytotoxic phenotype can be dissociated from this surface phenotype by the strength of STAT3 signalling. This observation could have several consequences. First, our observation suggests a cell of origin for human cytotoxic CD4+ T cells. This might also explain the ontogenic counterpart of large granular lymphocytes in leukaemia, which are often CD57+ and develop in states of chronic antigen stimulation (Lamy). Second, the decision between help and cytotoxicity appears to be part of a continuum, and while there are conditions that operate within the GC that appear to result in a bias away from cytotoxicity, relative to CXCR5+ cells that are found in the blood, there remains a small and measurable

cytotoxic subset within the TFH compartment. This might explain the paradoxical coincidence of antibody deficiency and autoimmunity which is observed with some forms of primary immune deficiency. Mutations that result in enhanced TFH formation might in some GCs also result in expansion of the cytotoxic subset. Indeed, this paradoxical association is observed in patients with *STAT3* GoF mutations.

Finally, our observations might have therapeutic potential. There is already overwhelming evidence that interruption of ligation of PD-1 can enhance anti-tumour responses, and alter the natural history of several types of cancer (Brahmer et al., 2012; Topalian et al., 2012). At present, it remains unclear whether this action is attributable to CD4+ T cells. In the light of our findings, monitoring CRTAM expression within CD57+ cells, PD-1+ compartment might be a useful strategy for assessing cancer immunotherapy. Beyond this, our observations raise the possibility that at least some of this effect might be the result of induction of cytotoxicity within CD4+ T cells. If this were the case, the action would be predicted to be even greater with concurrent use of treatments that enhanced STAT3 signalling.

CHAPTER 7 OVERALL SUMMARY AND DISCUSSION

The signals involved in shaping the faith of these subsets and their terminal differentiation include strength and duration of the TCR signalling, presence of specific costimulatory or co-inhibitory molecules as well as the presence of specific cytokines that mediate their signalling through a specific STAT molecules shape the faith of the T cell lymphocytes in assuming one pathway or another. Similarly CD8 T cells differentiate into different subset; naïve, Tcm, Tem and Temra , and this differentiation process is influenced by similar factors including strength and duration of TCR signalling, cytokines and their STAT molecules as well as mTOR. Most of the available information are based on animal studies.

The information gained from animal model is invaluable to understand paradoxical association of immunodeficiency that might also present as autoimmunity and

inflammation. However, the presence of rare human with specific mutations is crucial to confirm or refute the importance of a proposed pathway or mechanism that is also shared by other common autoimmune or inflammatory diseases in human. We aimed to elucidate mechanism of such diseases using two approaches.

First, a discovery project in which we identified a proband who presented with both inflammation and recurrent infections, including bronchiectasis by the age of 4 years old associated with hypergammaglobulinaemia. Whole exome sequencing revealed novel mutations in *STAT4* and *MTOR*.

Analysis of the whole exome sequencing, cellular phenotype and the functional analysis has shown that $STAT4^{P450S}$ is a novel and cytokine dependent mutation in the DNA binding domain (Takezaki et al. 2012; Yamazaki et al. 2014). Most of the reported DBD mutation in STATs molecules were gain of function(Faitelson et al. 2014) , (Sampaio et al. 2013) and (Uzel et al. 2013) and (Frans et al. 2014; Takezaki et al. 2012; Yamazaki et al. 2014; Sampaio et al. 2013). Similarly, $STAT4^{P450S}$ is a gain of function with prolonged phosphorylation (Figure 3.7 A-D) and binding to the nucleus (Figure 3.7 E). The prolonged binding was associated with prolonged transcription of *IRF1* (Figure 3.8 A-D) explaining the excessive formation of IFN-γ, T-bet up regulation and Th1 formation (Figure 3.9 B). We also observed enhanced IL-21 expression in cTFH cells, as a result of *STAT4* gain of function (Figure 3.10). This was also replicated in the *in-vitro* system of inducing TFH using IL-12 as a stimulus (Figure 3.10-E-H). Our findings provided the first *in vivo* evidence for the importance of STAT4 for human Th1 (IFN-γ), T-bet up regulation and TFH formation.

We have also discovered a novel $\boldsymbol{MTOR^{T2446S}}$ mutation in the highly conserved negative regulatory domain. The dysfunction of which was associated with constitutive active mTOR as assessed by level of pS6. So far there is no reported human germ line mutation in mTOR though there are reports of *PI3KCD* mutations (Angulo et al. 2013; Lucas et al. 2014) that were shown to cause downstream activation of mTOR. The clinical and cellular feature in this cohort is similar to our

proband and carrier of $MTOR^{T2446M}$ in terms of expansion of CD8+ T cell effector subsets (Temra) and IFN-γ production. The $\boldsymbol{MTOR}^{\mathbf{T2446S}}$ mutation as well is a gain of function that is causing activation of mTOR at the basal level. It is known that mTOR is important in CD4 and CD8 effector T cell formation. Moreover, mTOR cross talk with STAT4 giving rise to effector CD8 expansion. This cross talk of $\boldsymbol{MTOR}^{\mathbf{T2446S}}$ and $\boldsymbol{STAT4}^{\mathbf{P450S}}$ can explain the extreme deviated effector T cells of Th1 phenotype (IFN-g) and T-bet expression even at the mucosal surface seen in the patient.

The second approach we adopted to elucidate mechanism of the paradoxical association is through examination of T cell differentiation in patients with known defects such as in STAT3. *STAT3* LoF mutation result in autosomal dominant hyper IgE syndrome (AD-HIES) in which these patients present with unexplained atopic manifestations and extreme elevation of IgE. On the other hand, *STAT3* GoF mutations have been reported in which antibody deficiency occurs concurrently with organ-specific autoimmunity.

STAT3 loss of function (LoF) mutations is now well-characterised syndrome that results in Th17 deficiency in human. The pathophysiology of the high level of IgE in HIES, however, remains enigmatic. The level of IgE does not become high until patients reach 1 year of age(Kamei and Honig 1988). Interleukin- 4 (IL-4) response as a main Th2 cytokine is a well know inducer of class switching to IgE (Romagnani 1994; Avery et al. 2008). IL-10 is not readily expressed by recently formed Th1 or Th2 cells. IL-10 produced by CD4+ Th2 cells has also been shown to control excessive Th2 and IL-4 immunopathology in animal models. Formation of this regulatory subset was shown to be enhanced by STAT3-dependent cytokines. We have demonstrated here that there is a basal IL-10 production by CD4+ T cells that is STAT3 independent. There is also an inducible component of the IL-10 by Th2 cells, which is STAT3-dependent in human, similar to the mice data. We propose here, the deficiency in this inducible Treg phenotype in Th2 CD4+ T cells is one of the reasons toward the chronic Th1 immunopathology seen in the AD-HIES.

TFH cells express markers such as PD-1(exhaustion marker) with or without CD57 (cytotoxic marker) among other markers. Formation and function of TFH and cTFH are comprised in patients with AD-HIES (*STAT3* LoF) defined by the classical marker of CXCR5. However, it is not clear if there is a clear cytotoxic or exhaustion distinction between the two subsets based on expression of CD57 by TFH and cTFH. Moreover, there is no data to implicate STAT3 in the formation of these two subsets. We have presented evidence that CD57 expression identifies cells with cytotoxic potential, and a subset of these cells demonstrates overt cytotoxic activity and this cytotoxicity is STAT3 dependent. Indeed, STAT3 appears to be important for TFH to form in the first place, as CXCR5+ cells are less abundant in patient with *STAT3* LoF, and as we show here, more abundant in patients with *STAT3* GoF. Nevertheless, within the TFH population, STAT3 refractoriness occurs, and this is marked by CD57 expression, high level of PD-1, that coincides with adoption of potential for cytotoxicity.

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